CHEMICAL REVIEWS

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Review

Medicinal Chemistry of Isocyanides

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ABSTRACT: In eons of evolution, isocyanides carved out a niche in the ecological systems probably thanks to their metal coordinating properties. In 1859 the first isocyanide was synthesized by humans and in 1950 the first natural isocyanide was discovered. Now, at the beginning of XXI century, hundreds of isocyanides have been isolated both in prokaryotes and eukaryotes and thousands have been synthesized in the laboratory. For some of them their ecological role is known, and their potent biological activity as antibacterial, antifungal, antimalarial, antifouling, and antitumoral compounds has been described. Notwithstanding, the isocyanides have not gained a good reputation among medicinal chemists who have erroneously considered them either too reactive or metabolically unstable, and this has restricted their main use to technical applications as ligands in coordination chemistry. The aim of this review is therefore to show the richness in biological activity of the isocyanide-containing molecules, to support the idea of using the isocyanide functional group as an unconventional pharmacophore especially useful as a metal coordinating warhead. The unhidden hope is to convince the skeptical medicinal chemists of the isocyanide potential in many areas of drug discovery and considering them in the design of future drugs.

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1. INTRODUCTION

"Ishi no Ue nimo Sannen. . .". This is a typical Japanese proverb which can be literally translated as "on a stone for three years. . ." meaning that perseverance will win at the end. We feel that this ancient wisdom suits perfectly the medicinal chemistry of isocyanides. Indeed, while isocyanides were considered barely more than an oddity at the beginning of the XX century, the latest generation of chemists has started to include them in virtually every applied field involving molecular design, and nowadays the time is ripe for their full exploitation in medicinal chemistry.

In 1950, the discovery of the potent antibiotic compound xanthocillin (**1**)[1] from the broth of *Penicillium notatum*, whose structure was solved in 1956 by Ilse Hagedorn,[2] violated the

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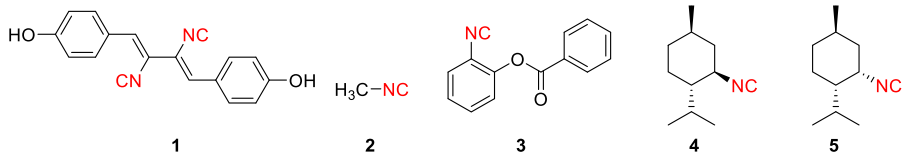

Figure 1. Examples of isocyanides.

taboo of the presence of isocyanides in natural products. Immediately after, it followed the seminal work of Professor Lamberto Malatesta, who reported for the first time a tetraalkyl isocyanide complex with a zerovalent nickel[3] marking the beginning the modern isocyanide coordination chemistry. Thanks to the discovery of the Ugi reaction in 1959,[4] the isocyanide moiety achieved over the following decades the status of an important reactive functional group in organic and combinatorial chemistry, and today it is widely employed in both photocatalysis and transition metal chemistry.[5−7] It is foreseeable that the electro-invisibility of isocyanides in electrochemistry will be surely explored and exploited in the near future.[8] The identification of methyl isocyanide (**2**), in interstellar clouds,[9,10] associated with the idea of being a fossil molecule which might have participated in the reactions at the basis of origin of life,[11,12] expands the potentiality of this fascinating and unique functional group in organic chemistry. Our recent findings have shown that, in specific molecular frameworks, the isocyanide group is metabolically stable.[13] Finally, the use of this functional group as a probe in biorthogonal reactions[14] qualifies it as an unusual pharmacophoric group (according to the definition given by Prof. Ryan Shenvi). Unfortunately, isocyanides do not have a good reputation among the majority of chemists, because of their atrocious odor. However, while the repellent smell issue is real for volatile low molecular weight isocyanides (from 1 to 10 carbon atoms), higher molecular weight isocyanides are generally odorless and chemically stable. Indeed, the human nose can hardly or at all detect a molecule containing more than 15 carbon atoms. Interestingly, the unpleasant odor of light molecular weight isocyanides is lost with efficient steric hindrance, such as in the case of 2-isocyanophenyl benzoate (**3**).[15] It has been shown recently that, by mixing an aromatic isocyanide with iodoperfluorobenzene, a solid adduct is formed by halogen bond, reducing the detection of its odor threshold by 50-fold, leaving unaltered the reactivity of the functional group.[16]

It is worth noting that even some liquid, low molecular weight isocyanides with less than 15 carbon atoms do not stink. For example, in one of our medicinal chemistry programs, we synthesized the two epimers of isocyano-menthol (**4** and **5**). While both compounds were oils, they displayed a delicate smell of grapefruit! (Figure 1).

In this review article, we aim to fill in an existing void associated with the use of isocyanides in medicinal chemistry, advocating for their renaissance supported by the vast amount of literature reporting them as structural moieties of naturally occurring products, and their associated biological activity in living systems. To the best of our knowledge, this is the very first review on the medicinal chemistry of isocyanides. However, we would like to provide the readers essential literature covering other aspects of this functional group, which has been a key inspiring source in the writing of this article.[17−23]

2. CHEMICAL FEATURES OF THE ISOCYANIDE GROUP

2.1. Structural Features

The isocyanide is the most strange, fascinating, and unique functional group in organic chemistry. Its chameleonic nature allows for its carbon atom to be virtually the subject of all reactivities in organic chemistry. Indeed, it can act as a nucleophile attacking activated electrophiles, as an electrophile being intercepted by different nucleophiles, as a carbene involved in formal [4 + 1] cycloaddition, and as a radical acceptor to form imidoyl radicals reaction intermediates.[6,7] Finally, the presence of a lone pair on the terminal carbon atom accounts for its strong metal coordinative properties allowing for the preparation of an endless number of coordination complexes.[24]

The strong coordinative nature of isocyanides is probably the most important feature displayed by isocyanide containing compounds in order to exert their biological activity. Because of all the described properties of isocyanides encompassing a wide range of chemical applications, one of the authors (G.C.T.) would like to paraphrase a sentence taken from the legendary organic chemistry textbook by Morrison and Boyd (his first one, read at the age of 20 years old) by stating that "if a chemist were allowed to choose ten aliphatic compounds with which to be stranded on a desert island" today he could decide to pick isocyanides instead of alcohols.[25]

With a length of only 1.167 Å, the small isocyanide is a linear functional group which can exist in two main resonance forms. Computational studies indicated that the carbenoid form is the most stable (Figure 2).[26]

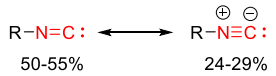

Figure 2. Two most important resonance forms of isocyanide account for the 84% of the weight of all resonance forms.

With the same spatial size with respect to nitrile, the isocyanide shares a very similar dipole moment (e.g., phenyl isocyanide, 3.44 D; benzonitrile, 4.51 D; methyl isocyanide, 3.83 D; and acetonitrile, 3.92 D),[27] an important similitude and feature a medicinal chemist should consider. Indeed, although isocyanides compounds are lipophilic, their large dipole moment aids in formation of favorable interactions in the protein binding pocket and enhances interactions with charged metalloproteins. Nitriles are poor coordinating groups in metal complexes, while isocyanides are strong chelators. Along these lines, it has also been suggested that that faint fruity odor of acetonitrile, opposed to the vile and atrocious odor of methyl isocyanide, is due to the strong ability of isocyanides to form complexes with metal ions in olfactory receptors.[28,29]

Not only can the isocyanide group act as a potential dipole, but it has been shown it can form hydrogen bonds at the

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carbon terminal atom. For example, phenyl isocyanide forms a stable hydrogen bond with alcohols, and this was demonstrated both via IR and NMR spectroscopy.[30,31] The formation of hydrogen bonds has also been observed between isocyanides and chloroform, water, phenols, and even with phenylacetylene.[30−32] It is important to highlight that the formation of such a hydrogen bond does not activate the isocyanide moiety for a nucleophilic attack and therefore they could be useful in protein binding interactions.

From a chemical perspective the isocyano group is isoelectronic with the carbon monoxide group, however displaying marked differences in the nature of the coordination bonds as we will see later.

It has been shown recently that, after coordination with a metal, both the nitrogen and the carbon atom of isocyanides can act as π-hole acceptors to the electron lone pairs of various kinds of atoms.[33,34] In the same article, the authors propose that the interaction with the nitrogen atom of isocyanide is more electrostatic and longer, while the interaction with the carbon atom of isocyanide is predominantly a charge-transfer complex which requires shorter distance in order to favor the n-π^* electron transition. This interesting interaction should not be missed in medicinal chemistry, whenever an isocyanide acts as coordinative group with a metal ion present in an active site (Figure 3).

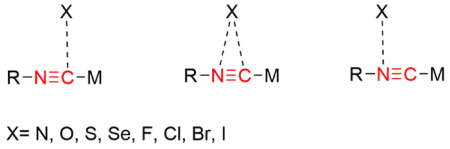

Figure 3. Different type of π-hole interactions of metalated isocyanides (formal charges were omitted).

2.2. Reactivity

Notwithstanding the intrinsic reactivity of the isocyano group as delineated above, isocyanides are inert at physiological pH toward water, thiols, alcohols, and amines, the most common nucleophilic groups present inside a cell. The reaction with amines can only take place by means of catalytic activation by a metal and under increased temperature.[5−7] Reactions with electrophiles (carbonyl and imine groups) require activation by protons or other Lewis acids. Below pH 5, isocyanides are hydrolyzed to formamides and the mechanism of hydrolysis has been debated.[35,36] Under basic conditions the scenario is strikingly different. Although aromatic isocyanides are susceptible to be attacked by hydroxide ion,[37,38] both aliphatic and aromatic isocyanides are in general able to withstand strong alkaline conditions. Indeed, our group successfully employed isocyanides under the strongly basic Bargellini reaction conditions without observing the formation of the amine component.[39]

2.3. Coordinating Properties

As previously anticipated, the isocyano group has strong coordinating properties. The carbon atom is usually providing the coordination with the metal atom. However, complexes where both nitrogen and carbon are engaged, as well as ones where one isocyanide engages two vicinal metals, have been reported (Figure 4).

Regarding the interaction with the metal, aliphatic isocyanides are markedly more σ-donors and weaker π-

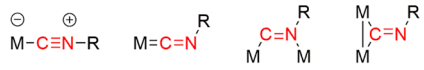

Figure 4. Metal-isocyanide bonding interactions.

acceptors than carbon monoxide, and this reflects their ability to form complexes with different oxidation states of the metals.[24] When the isocyano group is directly linked to a phenyl moiety, the extended conjugation provided by the aromatic ring allows for a better delocalization for the back-donating electrons, making aromatic isocyanides better π-acceptors than aliphatic ones.

As a confirmation, when electron withdrawing groups are present on the phenyl ring this property is enhanced.[40,41] When the back-donation prevails, the linearity with the metal is lost and such complexes display a dihedral angle of 130−140° between the metal and the isocyano ligand. In comparison, a standard metal methylisocyanide complex is linear with a dihedral angle of 180°.[42] To put it in a nutshell, the σ donor of isocyanide is the lone pair on the terminal carbon atom, while the π-acceptor is the unoccupied antibonding 2 π^* which can overlap with the occupied d orbitals of the metal. It is intriguing that, by varying the nature of the alkyl/aryl substituent, it is possible to switch the electronic features of isocyanides toward either carbon monoxide-like or cyanide anion-like behaviors.

Unlike carbon monoxide and its strong backdonation leading to a quasi-irreversible bond with the metal, isocyanides are able to dissociate when the backdonation is moderate.

As a rule of thumb, it is easy to remember that aromatic isocyanides form stronger bonds with the metal compared to aliphatic ones because of their enhanced backdonation (e.g., phenyisocyanide is a stronger ligand than methylisocyanide; Figure 5).[43]

Figure 5. Old vs new representation of a metal-isocyanide bond.

It is important to highlight the notable differences between carbon monoxide, cyanide anion, and isocyanides in the formation of iron complexes. CO binds exclusively to ferrous (Fe II) heme with an affinity 220 times greater than oxygen and a half-time between 200 and 360 min.[44,45] On the contrary, the cyanide ion has a preference for binding the ferric (Fe III) heme (methemoglobin) and other cytochromes with ferric ion (e.g., cytochrome c oxidase). Isocyanides lay halfway between CO and CN^-, and according to the nature and steric bulk of R, they can interact only with specific iron proteins, either with ferric or ferrous heme. The nature of the metal also plays a decisive role on the quality of the backdonation. Usually, metals of group IB and IIB like copper, silver and zinc form essentially only σ-bonds with isocyanides, resulting in unstable complexes with high dissociation propensity. On the other hand, iron can actively participate to the backdonation forming stable complexes. However, one should keep in mind that the interaction between the metal and the isocyano group can alter its reactivity, as discussed earlier. Actually, two situations are possible where ligands can be (i) innocent, where the isocyano group is unreactive, and complexes of this type are indefinitely air-stable, and (ii) noninnocent, where an

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activated isocyano group will react with nucleophiles (alcohols, amines, and thiols).[46] In the latter, the coordination with the metal has changed the electron density on the isocyanide carbon atom, which is now prone to insertion reactions via nucleophilic attack. Infrared analysis can reveal the innocent vs the noninnocent scenario. Upon spectra collection, it is possible to calculate the result of the related stretching peaks from the following equation:

$$\Delta\upsilon(\mathrm{NC}) = \upsilon(\mathrm{NC})_{\mathrm{coord}} - \upsilon(\mathrm{NC})_{\mathrm{free.}} \quad (1)$$

A value greater than 40 cm^{-1} indicates that the isocyanide can be intercepted by nucleophilic agents (Figure 6).[47,48]

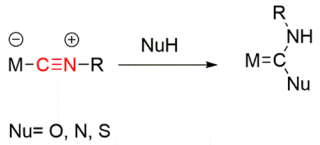

Figure 6. Metal-assisted isocyanide insertion.

This increased reactivity of the metal-coordinated isocyano group could be exploited in medicinal chemistry by employing the isocyano moiety as a strategic group for the design of covalent inhibitors directed to the active site against a wide range of metalloproteins.

3. ISOCYANO GROUP IN NATURAL PRODUCTS

After the identification of xanthocillin in the fifties, hundreds of secondary metabolites containing the isocyano group have been isolated both from prokaryotes (bacteria and cyanobacteria) and eukaryotes (fungi and marine sponges). It is interesting to note that the majority of the isocyanide metabolite producer organisms are sponges and cyanobacteria. Sponges are primitive organisms originated around 500 000 years ago, with fossils dating back to the Precambrian, while cyanobacteria are older, with fossil records unambiguously dated at 1.89−1.84 Ga.[49] Although, the ecological role of the said metabolites is barely known, many of them have been shown to possess potent antibacterial, antifungal, and antiprotozoal activities when tested. Isocyanoterpenes have also been found on the skin of nudibranchs, brightly colored marine mollusks devoid of a protective shell. Interestingly, it has been shown nudibranchs are not able to synthesize these compounds on their own, but they acquired them through their diet from specific sponges.[50,51] It is reasonable to ascribe a defensive role to these metabolites, since many isocyanoterpenes possess ichthyotoxicity. Indeed, predators for nudibranchs are quite rare.[52,53] Although it is generally believed that plants are not able to synthesize isocyanides, a 2012 publication reported the identification of the isocyanide named isocyalexin A (**6**) produced by Rutabaga roots (*Brassica napus*, Figure 7), as the first phytochemical isocyanide.[54] It should be noted that the authors were able to isolate only tiny amounts of this compound from the rutabaga roots, therefore being unable to provide a full characterization at first. However, once they exposed to UV-light for 90 min rutabaga root slices, compound **6** was isolated in higher quantities and finally fully characterized. Compound **6** showed antifungal activity by inhibiting the mycelia growth, especially against the plant pathogenic fungi *Rhizoctonia solani*,[54] suggesting the possible ecological role played by this isocyanide. In a following publication, the study of the biosynthesis of **6** unveiled its corresponding *O*-sulfatated aldoximes as the intermediate which undergoes a Beckmann rearrangement resulting in the formation of the isocyano group.[55] Formation of isocyanides via Beckmann rearrangement of activated aldoximes has already been reported in literature.[56,57] Although this is the only article to date reporting the occurrence of isocyanides in plants, it can serve as a source of inspiration for phytochemists aiming for the finding of novel secondary metabolites.

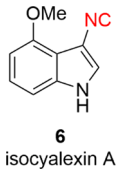

Figure 7. Isocyalexin A.

3.1. Biosynthetic Origin and the Ecological Role of Isocyanide

Several studies have shown the presence of at least three different operating mechanisms used by cells to graft the isocyano group in a secondary metabolite. Contrary to the simpler assumptions: (i) the cyanide ion is not the sole group involved in the formation of the isocyano moiety; (ii) the classic synthetic workbench procedure for the synthesis of isocyanides (that is amine → formamide → isocyanide), is not at work in Nature. Although in many cases isocyanoterpenes are also accompanied by the corresponding formamides, landmark was the work of Professor Giulio Sodano in Naples using a ^{14}C of the formamide axamide-1, the precursor of the isocyanide axisonitrile-1 previously isolated by the sponge *Axinella cannabina*. Indeed, after 5 days of feeding with the radioactive formamide, no radiolabeled isocyanide was detected, but only ^{14}C-axamide-1. This experiment proved that formamides are not the precursor of isocyanide but are the result of decomposition of isocyanide.[58] Similar results were later obtained using the radiolabeled formamide of 2-isocyanopupukeane. Even in this case no detection of the related isocyanide has been detected.[59]

The first general biosynthetic pathway is characterized by a late-stage functionalization, with the insertion of the isocyano group at the end of the synthesis. This is a common case scenario for the terpene-isocyanides of marine origin. This pathway was shown for the first time by Professor Mary Garson, unveiling the biosynthesis of 7,20-diisocyanoadocian, a terpene isolated from the *Amphimendon terpenensis* sponge. In these organisms, hydrogen cyanide is formed by the action of glycine dehydrogenase decomposing glycine in HCN and CO_2. Hydrogen cyanide intercepts the generated tertiary carbocation of sesquiterpenes reacting in a stereocontrolled way through an enzyme assisted mechanism.[60,61] It is possible that these sponges have developed cyanide resistant respiratory systems in order to survive the highly toxic cyanide ion (Figure 8)

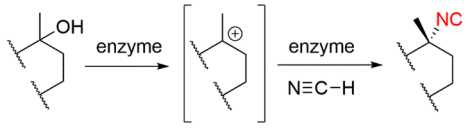

Figure 8. Typical mechanism for the formation of marine isocyanides.

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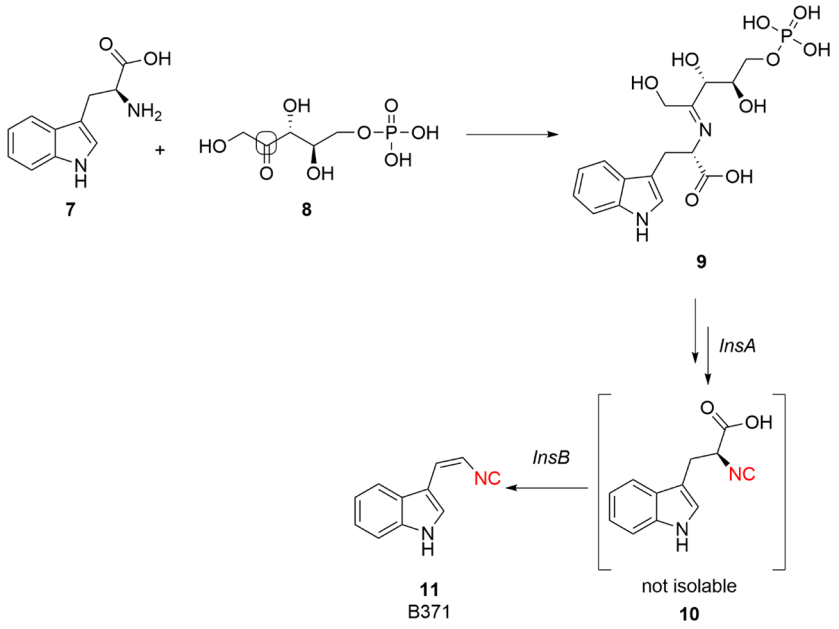

Figure 9. Typical biochemical pathway for the synthesis of terrestrial isocyanides.

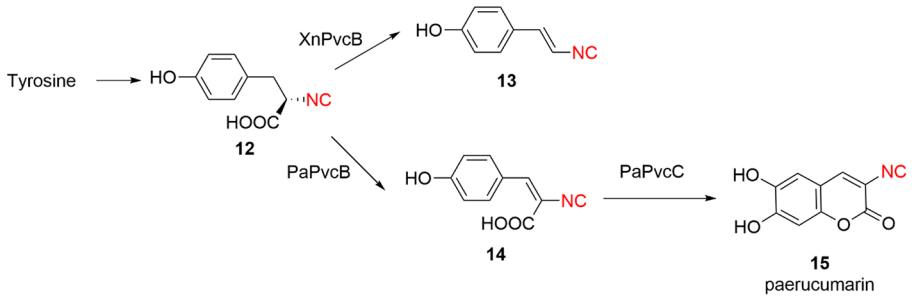

Figure 10. Biosynthetic routes of tyrosine derived isocyanides.

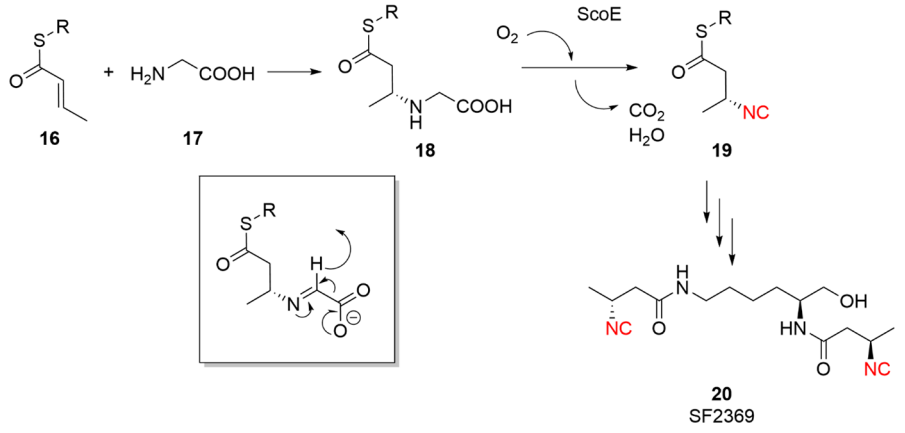

Figure 11. Biochemical pathway for the synthesis of isocyanides produced by *Actinobacteria*.

Further studies confirmed the same synthetic pathway for the 2 and 9-isocyanopupukeanane and for the kalihinol F.[59,62] The thiocyanate group also plays a great role as precursor of marine isocyanides. Indeed, while some thiocyanoterpenes derive from isocyano analogues, it has been demonstrated that for sponges of genus *Axynyssa* the reversed pathway is at work (from isothiocyanoterpene to isocyanoterpene).[63]

The second general biosynthetic pathway is characterized by an early stage functionalization where the formation of the isocyano group derives from the nitrogen of amino acids, especially tyrosine and tryptophan, followed by an iron-mediated decarboxylation. Further diversification reactions increase the structural complexity. This pathway occurs mainly in fungi and bacteria. Historically, early studies on xanthocillin allowed for the discovery of this path by feeding *Penicilllum notatum* strains with tyrosine-2-^{14}C−^{15}N and in turn isolating ^{15}N xanthocillin.[64] Recently two genes involved in the synthesis of the indole antibiotic B371 (**11**) named *isnA* and

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isnB revealed the origin of the isocyano group. These two enzymes are generally known as isonitrile synthases (ISNs) or isocyanide synthases (ICSs), and they can convert the amino group of aromatic amino acids into an isocyanide. Surprisingly, the carbon atom of the isocyanide derives from the C-2 carbon atom of ribulose-5-phosphate (**8**)[65,66] and the two key mechanistic steps are the imine formation between the keto group of ribulose and the amine of tryptophan (**7**), and the decarboxylative step mediated by an iron containing enzyme (Figure 9).

Similar enzymes named PvcA and PvcB are involved in the synthesis of tyrosine derived isocyano compounds, and studies for the decarboxylative step by the PvcB enzyme have been reported.[67,68] The enzyme PvcB is like the InsB enzyme a α-ketoglutarate dependent oxygenase. In the case of the compound paerucumarin (**15**) a third gene PvcC is involved. It catalyzes the conversion from the open form to paerucumarin by hydroxylation-oxidation and cyclization (Figure 10).[68]

Several other related genes implicated in the mechanisms of isocyanide formation in fungi, bacteria, and cyanobacteria have been identified from other species.

The third general biosynthetic pathway for the formation of the isocyano group has been reported in the synthesis of natural product SF2369 (**20**) from *Actinobacteria*. In this case the isocyano group solely derives from glycine. The nonheme iron(II) α-ketoglutarate decarboxylase ScoE catalyzes the formation of the isocyano group through an oxidative decarboxylation.[69] Recent evidence indicates that after dehydrogenative formation of the imine, the decarboxylation proceeds via enzyme mediated H-abstraction[70] (Figure 11).

Do note that, due to the abundance of such gene orthologues in other species, it is foreseeable to expect future discoveries of several isocyanide containing natural products.[68,71−73]

Beyond isocyanide synthases, many microorganisms have acquired the ability to degrade the isocyano group to formamide by using a family of enzymes called isocyanide (isonitrile) hydratase. To date, three of those enzymes have been characterized: one obtained by *Pseudomonas putida*, one from *Streptomyces thioluteus*, and the other by *Arthrobacter pascens*.[74−76] The said enzymes are inducible, and they can also transform simple synthetic isocyanides such as cyclohexyl-, benzyl-, pentyl, *tert*-butyl isocyanide, methyl-, and ethyl-isocyanoacetate. Interestingly, it was shown that isocyanides can be converted to amine by a nitrile hydratase from *Rhodococcus*.[77]

Although it is impossible to know all the ecological roles associated with every isocyanide Nature has synthesized, researchers have found the main function for some of them. For example, some insect-pathogenic Gram negative bacteria (*Xenorhabdus nematophila* and *Photorhabdus luminescens*) synthesize and display on their cell surface rhabduscin (**21**), an amidoglycosil vinyl isocyanide, which has been shown to inhibit the enzyme phenoloxidase at low nM digits. This enzyme is critical for the innate immune system of insects as it is responsible for the production of the hard polymeric compound melanin, which can trap microbial pathogens. A similar behavior has been described for byelyankacin (**22**) isolated from *Enterobacter* species, which is able to inhibit phenoloxidase as well (Figure 12).[78]

This inhibitory action has been reported both on insect phenolooxidases as well as mushroom and human tyrosinases.

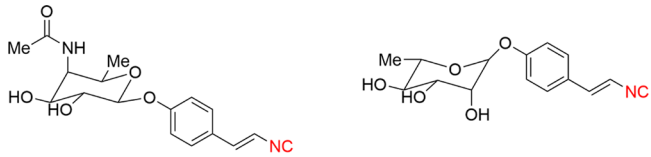

Figure 12. Chemical structure of rhabduscin (**21**) and byelyankacin (**22**).

Interestingly the aglycone part of rhabduscin (**21**) maintains its activity, and it is the most potent tyrosinase inhibitors[78] (Table 1). Beyond their natural role, these compounds can be used as skin-whitening agents as they are by far more active than kojic acid, a successful commercial cosmetic skin whitening. The replacement of the isocyano group with a nitrile in this family of compounds, is associated with the complete loss of inhibitory activity toward the copper containing enzyme tyrosinases for this family of compounds. It is therefore plausible to envision an inhibitory mechanism of action enabled by the isocyano functional group, acting as a copper-coordinating moiety.

Finally, paerucumarin **15** is responsible for two roles. It regulates the events in the biofilm synthesis and facilitates the induction of bacterial siderophores in *Pseudomonas aeruginosa*, chelating and reducing the iron levels within the extracellular environment.[79]

3.2. Isocyanides in Fungi and Their Biological Activity

As discussed in the introduction, the very first isolated natural isocyanide was xanthocillin X (**1**), a deeply yellow, light sensitive compound discovered in 1950 by Rothe from the mold of *Penicillum notatum* Wrestling.[1,80,81] Later related analogues were isolated, displaying point variations for the substituents on the aromatic rings, such as xanthocillins Y1 (**23**) and Y2 (**24**; Figure 13).

Early studies on the metabolic origin of xanthocillin X demonstrated that the isocyanide nitrogen atom derives from tyrosine.[64] Unsurprisingly, xanthocillin X proved to possess a potent antibiotic activity against both Gram positive and Gram negative bacteria, as well as against yeasts and fungi, inducing slow onset of bacterial resistance in serial passages (Table 2).[82−84] It was sold for more than 15 years under the commercial name of Brevicid and used as topical antibiotic.[84] It showed a synergistic effect with the antibiotic tyrothricin[85] and this mixture was used topically.[86]

Recently, the genes involved in its biosynthesis were identified in the fungi *Aspergillus fumigatus*, a well-known opportunistic human pathogen. Interestingly copper deprivation causes an increased transcriptional activity of these genes, indicating a pivotal role for xanthocillin X in maintaining copper homeostasis. Xanthocillin X acts then as a chalkophore to bind copper in the environment and transport it back inside the fungus for its employment in copper-dependent enzymes. Four isocyanide synthases were identified within the fungus genome and four xanthocillins derivatives were abundantly produced, while in the presence of copper their production was highly reduced. More specifically, the enzyme named XanB converts tyrosine in intermediate **14**, and then XanG catalyzes the oxidative dimerization to give xanthocillin X. XanG is a cytochrome P450 monooxygenase, homologue of the yeast enzyme Dit2, which is involved in the dimerization of tyrosine resulting in *N*,*N*-bisformyl dityrosine, a constituent of the

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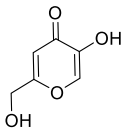
Table 1. IC_{50} Value for the Inhibition of Mushroom Tyrosinase and Phenoloxidase from *Galleria mellonella* (Waxmoth Larvae)

Compound	Tyrosinase inhibition (nM)	Phenoloxidase inhibition (nM)
21	15.1	64.1
22	37.1	184.9
13	7.9	61.7
 **Kojic acid**	22,173	ND

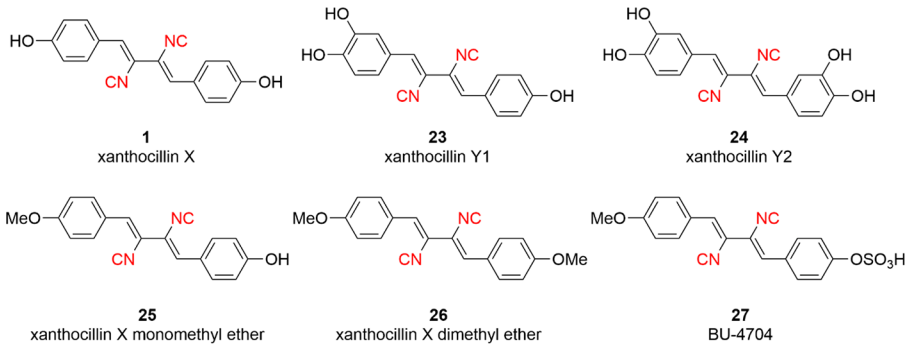


Figure 13. Xanthocillin X (**1**) and its derivatives.

Table 2. Partial Antibiotic Spectrum of Xanthocillin X (1)

microorganism	MIC (μg/mL)
E. coli	0.2
P. aeruginosa	0.2
B. subtilis	0.5
C. tetani	0.2
M. tuberculosis	5.0
C. albicans	4.0

Table 3. Partial Antibiotic Spectrum of BU-4704 (27)

microorganism	MIC (μg/mL)
E. coli	1.6
S. aureus	<0.1
B. subtilis	<0.1
K. pneumonia	0.4
P. aeruginosa	>50
C. albicans	>50

spore wall. Hydrolysis of the isocyano group to give the formamido analogue is mediated by the isocyanide hydratase XanA which can aid with the isocyanide detoxification.[87,88] Interestingly, the coculture of *Aspergillus fumigatus* and the bacteria *Streptomyces peucetius*, in competition for nutrients, stimulated the production of secondary metabolites such as the xanthocillin analogue BU-4704 (**27**) along with its diformamide analogue fumiformamide. Fumiformanide revealed strong cytotoxic activity (IC_{50} between 0.65 and 1.12 μM) against a panel of cancer cell lines.[89]

The biological activity of BU-4704 was previously reported indicating broad spectrum activity against Gram + and Gram− bacteria and fungi (Table 3). Furthermore, this compound was cytotoxic against human colon carcinoma cells (HCT-116) and murine melanoma B16−F10 with an activity of 0.63 μg/mL and 4.3 μg/mL.[90,91]

Recently xanthocillin X was isolated from *Penicillum commune* in a deep-sea sediment. The study of its antibacterial and antitumoral profile confirmed its strong antibacterial activity against *Staphylococcus aureus* (MIC = 2 μg/mL) and *Escherichia coli* (MIC = 1 μg/mL), together with being cytotoxic against several cancer cell lines (e.g., MCF-7, HepG2, MDA-MB-231, Du145, HeLa, and H460) with an activity between 7.0 and 12 μg/mL.[92] Further studies revealed that its cytotoxicity against HepG2 cancer cells is ascribed to a significant induced autophagy via a noncanonical signal pathway involving class III PI3K/Beclin 1 and MEK/ERK signaling pathways.[93] On the other hand, when a phenotypic screening method based on changes in cell morphology of *Penicillium oryzae* was carried out, xanthocillin X was shown to induce vacuolization, a signal of apoptosis. Possible biological targets might be complex V, PLC, and prostaglandin synthetases.[94]

To this point, a very recent publication disclosed the mechanism of action of xanthocillin X. At first, the authors confirmed the broad antibacterial activity of **1**, both on Gram + and − , in particular against *Acinetobacter baumannii* (MIC 0.25−0.5 μM), while being inactive against *Enterococcus faecalis*. Despite its ability to chelate copper(II), demonstrated via metal−ligand fluorescent titration, this seems not to be the mechanism responsible for its antibacterial activity. Indeed, xanthocillin dimethyl ether **26** is not able to chelate copper(II) but, in an *Acinetobacter baumannii* knockout strain of two efflux pumps, maintains antibacterial activity. Sequencing the xanthocillin-resistant strains of *Acinetobacter baumannii*, they were able to identify a mutation in the protein porphobili-

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Table 4. Antibacterial (MIC μg/mL) and Cytotoxic Activity (IC_{50} μM) of 1 and 23

	A. baumannii	*K. pneumoniae*	*P. aeruginosa*	*SF-268*	*MCF-7*	*HepG2*	*A549*
1	0.1	0.1	0.1	1.23	0.26	1.34	0.38
23	0.5	0.5	8	2.11	3.65	4.50	5.04

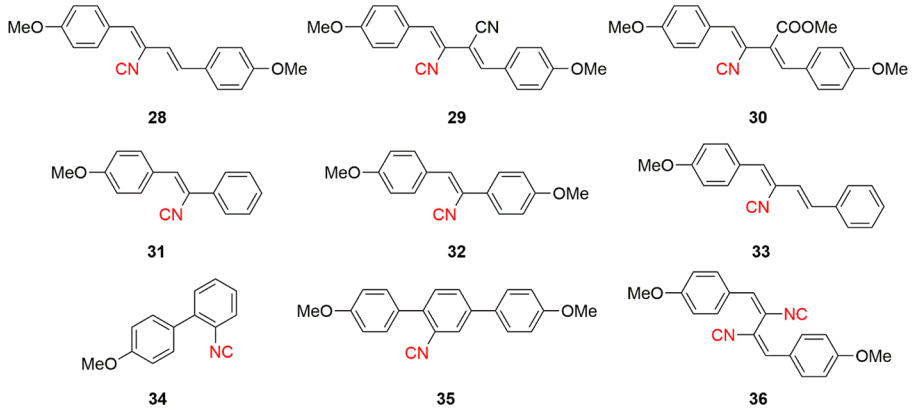

Figure 14. Simplified analogues of xanthocillin X dimethyl ether (**26**).

nogen synthase, the enzyme which catalyzes the formation of porphobilinogen from 5-aminolevulinic acid. Porphobilinogen is the key intermediate in the formation of all tetrapyrroles such as heme and the latter acts as negative feedback for the activity of the enzyme. They were able to demonstrate that xanthocillin X binds the iron atom of iron protoporphyrin X, sequestrating it. Reduced levels of heme leads to a misregulation in heme-dependent enzymes, including suppressed inhibition of porphobilinogen synthase. The net consequence is a high production and accumulation of porphyrins, thus generating toxicity.[95]

A series of metabolites, including xanthocillin Y1 (**3**), were isolated from an Antarctic derived *Penicillium chrysogenum* strain, with xanthocillin X (**1**) displaying dominant activity with a titer of 0.9 g/L (Table 4). These two compounds demonstrated significant cytotoxicity on four cancer cell lines, as well as antibacterial activity against Gram-negative bacteria (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumonie*) with a MIC of 0.125 μg/mL.[96]

Xanthocillin X monomethyl ether (**25**) was isolated from the fungus *Dichotomyces albus*.[97] This compound displayed wide antibacterial activity against Gram-positive and negative bacteria as well as against certain fungi. Cytotoxic activity on HeLa cells was also reported with a LD_{50} of 0.3 μg/mL as well as an in vitro antiviral activity against plaque formation of the Newcastle disease virus (NDV), which is responsible for an avian disease transmissible to humans. Unfortunately, the compound proved to be toxic on mice. The acute LD_{50} was 40 mg/kg, but at doses higher than 200 μg/mouse/day, a rapid decrease in body weight followed by animal death was recorded, and for this reason its development was discontinued. Its antiviral activity is to ascribe to the inhibition of protein synthesis in virus infected cells. In more detail, complete inhibition of the synthesis of hemagglutinin (HA) was reported 1 h after the treatment with xanthocillin X monomethyl ether.[98]

Xanthocillin X monoethyl ether was also shown to reversibly inhibit prostaglandin synthesis by blocking the conversion of arachidonic acid into endoperoxide prostaglandin H_2 at 20 μM.[99]

Xanthocillin X dimethyl ether (**26**) was shown to be efficacious against many solid tumors maintaining a low toxicity in mouse models. IC_{50} against several cancer cell lines have been reported (e.g., Lung Lewis carcinoma IC_{50} = 0.46 μg/mL/Meth-A, IC_{50} = 0.71 μg/mL). Peritoneal injection on Balb/c mice with a daily dose of 30 mg/kg for 4 consecutive days allowed for an 88.5% reduction of tumor growth. In the same patent, acute toxicity of xanthocillin X dimethyl ether was reported at 120 mg/kg.[100] Recently cytotoxicity of xanthocillin X dimethyl ether on other cancer cell lines has been reported, showing significant activity against A2780T (IC_{50} = 0.45 μM), Calu3 (IC_{50} = 0.52 μM), HepG2 (IC_{50} = 1.21 μM), MDA-MB-435 (IC_{50} = 1.78 μM), ACHN (IC_{50} = 6.87 μM), and RD (IC_{50} = 48.70 μM).[101] To note that the diformamide analogue of xanthocillin Y2, named cordyformamide has been isolated from fungus *Cordyceps brunnearubra*. Conversely to its parent compounds, this compound has only a modest cytotoxicity against human breast cancer (IC_{50} = 39 μM) and no cytotoxicity against other cancer cell lines (e.g., KB cells, NCI-H187) and Vero cells.[102] Xanthocillin monomethyl ether mimics the action of thrombopoietin (TPO) to the same extent, inducing cell proliferation of TPO sensitive human leukemia cell lines with an EC_{50} of 4.0 ng/mL.[103] A recent synthesis of xanthocillins[104,105] enabled the preparation of natural and unnatural xanthocillins and their evaluation as agonists on the thrombopoietin receptor (Figure 14).

While xanthocillin X and compound **34** were inactive, compounds **25** and **26** displayed activity. The *E,E* isomer of xanthocillin dimethyl ether (**36**) was also inactive, suggesting that the configuration *Z,Z* found in the natural products is essential for the activity. The removal of one isocyanide group (**28**), reduced the activity without suppressing it. Compound **29** was still active, while compound **30** was inactive. Compounds **32** and **33** were still active but with a reduced potency. Interestingly the light stable terphenyl derivative **35** showed the same potency of xanthocillin X dimethyl ether **26**.[106]

Xanthoascin (**37**) was isolated from *Aspergillus candidus*.[107] The compound is cytotoxic toward HeLa cells, but it was shown to be highly hepatic and cardiotoxic and teratogenic in animal models (Figure 15).

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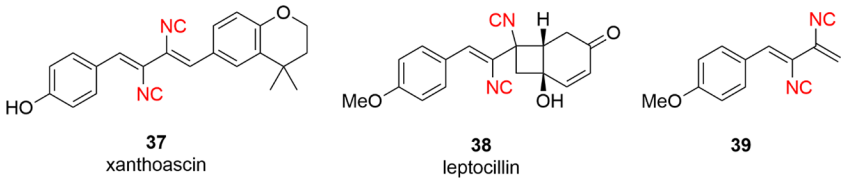


37 xanthoascin **38** leptocillin **39**

Figure 15. Xanthoascin (**37**), leptocillin (**38**), and its degradation product **39**.

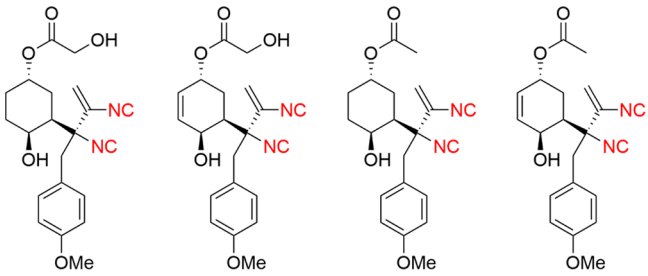
Interestingly, xanthoascin demonstrated activity against phytopathogens by displaying low inhibition levels (MIC = 0.31 μg/mL) against the bacteria *Clavibacter michiganense*. This bacterium is responsible for the potato ring rot, one of the most important seed potato diseases.[108]

Leptocillin (**38**; Figure 15; previously known as MK4588) was isolated by *Leptosphaeria*. This compound has a limited antibiotic activity and is moderately stable under neutral conditions but unstable under acidic and basic media. Under basic conditions, it is degraded to the more potent compound **39** (Figure 15 and Table 5).[109]

Table 5. Selected Antibacterial Activity (MIC μg/mL) of 38 and 39

compound	*S. aureus*	*B. anthracis*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
38	25	6.25	>100	1.56	>100
39	6.25	6.25	>100	0.78	>100

Recently, antibiotics related to leptocillin were isolated from the filamentous fungus *Ophiosphaerella korrae* and named MDN 0057/0058/0059/0060 (**40**–**43**; Figure 16). These

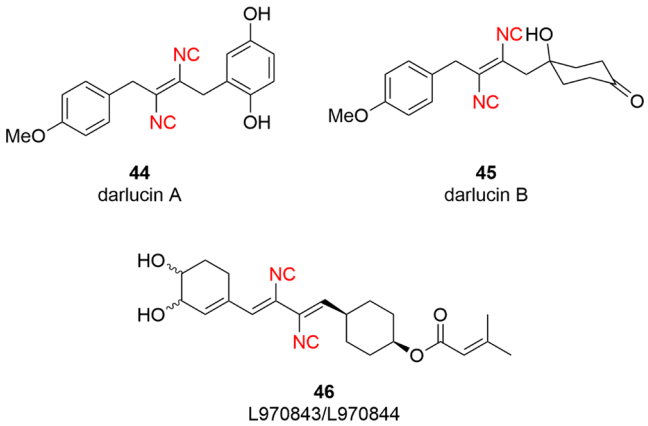


40 MDN 0057 **41** MDN 0058 **42** MDN 0059 **43** MDN 0060

Figure 16. Isocyanides isolated from *Ophiosphaerella korrae*.

compounds possess a potent antibacterial action against Gram negative bacteria with a MIC of 0.2 μg/mL against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli*. No antibacterial activity was observed against *Staphylococcus aureus* and on eukaryotic cells like HepG2 and Fa2N4. Although the mechanism of action is not fully elucidated, these compounds seem to act by inhibiting the cell wall synthesis.[110]

Other analogues of xantocillin named darlucin A (**44**) and B (**45**; Figure 17) were isolated from *Darluca filum*. In this case, these molecules feature a 1,2-diisocyanoalkene moiety instead of a bis-vinylen diisocyanide. This set of darlucin compounds was also characterized by a strong antimicrobial activity against several bacterial strains (MIC between 2.5 and 5 μg/mL), while displaying a weak cytotoxicity on several cancer cell lines (IC_{50} between 25 and 50 μM).[111]



44 darlucin A **45** darlucin B

46 L970843/L970844

Figure 17. Darlucin A (**44**), darlucin B (**45**), and L970843/L970844 (**46**).

From an unidentified fungal strain, two novel isomeric xanthocillin-like derivatives were isolated and named L970843 and L970844 (**46**). They displayed good activity against bacterial and fungal strains.[112]

From genus *Trichoderma*, a series of rather unstable and reactive cyclopentane isocyanides antibiotics have been isolated and named isonitrin and isonitric acids (**47**–**60**; Figure 18 and Table 6).[113−117] In these compounds an isocyanide moiety is grafted on a cyclopentane ring, along with the presence of one or more reactive epoxides or Michael acceptors. Interestingly, in the 1970s the ingestion of grass containing *Trichoderma* species has been associated with the ovine ill thrift, a sheep disease characterized by a slower growth rate of livestock. This phenomenon is to ascribe to the antibiotic action of these isocyanides against bacteria in the rumen involved in the cellulose digestion.[118−120] The biological effect of these isocyanides was blocked when nickel salts were added to broth culture, due to the induction of metal assisted isocyanide polymerization.

The chemical complexity of these low molecular weight isocyanides is noteworthy. For example, isonitrin C is characterized by six contiguous chiral centers. Isonitrin A appears to be the best antibiotic of this family, with a good activity against both Gram positive and negative bacteria, yeast, and fungi. *Pseudomonas aeruginosa* was not killed by these antibiotics. The acute toxicity of these antibiotics was also evaluated by injection into mice and LD_{50}s calculated (isonitrin A = 160 mg/kg; isonitrin B = 300 mg/kg; isonitrin C = 100 mg/kg; isonitrin E = 240 mg/kg; Figure 18).[121]

A novel cyclopentane isocyanide named MR304A (**58**) was isolated in 1995 from the culture broth of *Trichoderma harzianum*. This compound was shown to inhibit mushroom tyrosinase in a noncompetitive way with respect to tyrosine, displaying an IC_{50} of 7.5 μg/mL and a K_i of 40 μM. The compound was also able to inhibit the melanogenesis of B16 melanoma cells with an IC_{50} of 1.0 μg/mL.[122]

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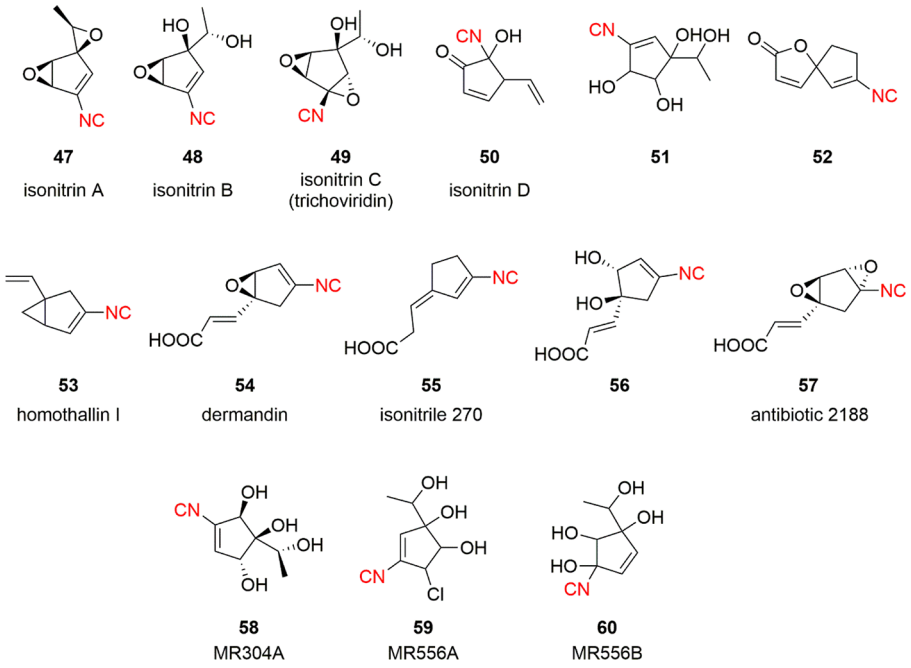

Figure 18. Isocyanides isolated from *Trichoderma* species.

Table 6. Selected Antibacterial-Antifungal Activity (MIC μg/mL) of 47−50 and 57

microorganism	47	48	49	50	57
S. aureus	1.56	200	25	>200	6.25
K. pneumoniae	6.25	>200	100	>200	50
P. aeruginosa	>200	200	100	>200	100
C. albicans	6.25	>200	25	12.5	>200
S. cerevisiae	3.13	50	25	ND	>200

Other compounds derived from *Trichoderma* (**55** and **59**) showed to be able to block the synthesis of melanin with an IC_{50} on mushroom tyrosinase of 1.72 and 47 μM, respectively. Furthermore, they were able to inhibit melanin synthesis in B16 melanoma cell with a MICof 0.1 and 2.2 μM and a cytotoxicity of 9.8 and 54.3 μM. Compound **55** was able to inhibit tyrosinase with an IC_{50} of 1.4 nM being thousands of times more active than kojic acid and hydroquinone (IC_{50} = 9.1 μM)[123] and to block the Pdr5P multidrug transporter in *Saccharomyces cerevisiae*, although high concentration were required (IC_{50} = 225 μM).[124]

The isocyanide natural product named A-32380A (**61**) was isolated from *Pyranochaeta*, a genus of fungal plant pathogen,[125] while the compound brassicicolin A (**62**) was isolated from *Alternaria brassicicola*,[126] a phytopathogenic fungus responsible for the black spot disease in *Brassica* species (Figure 19). A recent study has shown that brassicicolin A is responsible for the foliar lesions on crucifer plants.[127,128]

Both molecules derive from the chemical manipulation of mannitol. Notwithstanding the poor absorption of these polar compounds, the antimicrobial activity of these isocyanides is excellent with a broad activity against Gram positive bacteria. A-32390A was also fungistatic against *Candida albicans* with a minimal inhibitory concentration of 2.5 μg/disc while brassicicolin A was inactive against *Candida*. *Cryptococcus neoformans* and *Histoplasma capsulatum* were also killed by A-32390A but at higher concentration (MIC = 10 μg/disc).

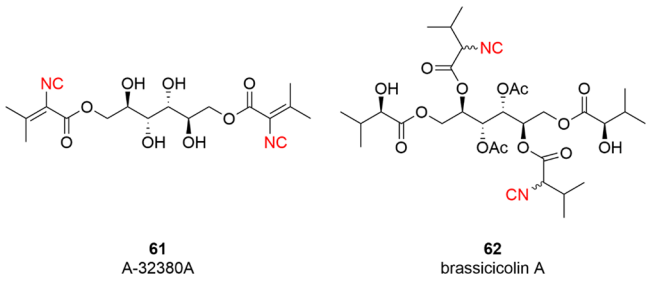

Figure 19. A-32380A (**61**) and brassicicolin A (**62**).

Furthermore, A-32390A was able to inhibit dopamine β-hydroxylase in a noncompetitive manner, a copper-containing protein, with an IC_{50} of 1.7 μg cm^{-3} and a K_i of 3 μM,[129] while displaying an acute toxicity (LD_{50}) on mice greater than 1 g/kg.[125]

Two novel isocyano metabolites closely related to brassicicolin A, named maculansins A (**63**) and B (**64**), were discovered from *Leptosphaeria maculans* (Figure 20).[130] No biological activity has been reported for these metabolites

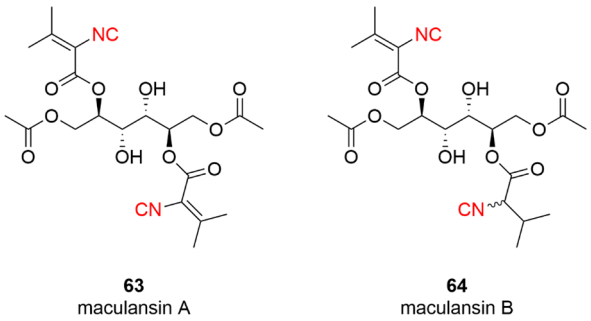

Figure 20. Maculansin A and B.

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3.3. Isocyanides in Bacteria and Cyanobacteria and Their Biological Activity

Bacteria and cyanobacteria produce many isocyano derived compounds, using the same biosynthetic pathways as fungus. The previously discussed paerucumarin (**15**) is a coumarin containing isocyanide, isolated for the first time from *Pseudomonas aeruginosa*.[66,68] The name derives from the contraction of *P. aeruginosa* and coumarin. The bacteria release this isocyanide in the surrounding environment.[79] There, it is involved in the generation of biofilm on solid surfaces. Paerucumarin is also able to bind iron ions without acting as a siderophore.[131] Its role is therefore to lower the environmental concentration of iron ions, inducing the production of *Pseudomonas aeruginosa* siderophores.

The compound named SF2768 (**65**) has been isolated from *Streptomyces thioluteus*. This compound contains two isocyanide moieties and it primarily acts as chalkophore and tyrosinase inhibitor, with an activity comparable with those of kojic acid.[132] The presence of the two isocyano groups is mandatory for the chalkophore action as the mono isocyanide derivative is 30-fold less active, while its diformamide analogue was completely unable to chelate copper. Copper ions are bound with a 1:2 ratio to SF2768. The activity of SF2768 against a range of bacteria is usually moderate (inhibitory rate of 60% at 100 μM) and it could be ascribed to its activity as chalkophore resulting in an unselective depletion of copper toward most microorganisms. This copper shortage translates into a reduced activity of copper dependent enzymes such as cytochrome *c* oxidase. As a comparison, the copper chelating activity of SF2768 has an EC_{50} = 82.2 μM with respect to an EC_{50} of 358.3 μM of EDTA. Other analogues of SF2768 have been identified (SF2768I, SF2768K, SF2768L) and probably they share a similar mechanism of action.[133] *Streptomyces thioluteus* also produces an isonitrile hydratase SfaF which serves as detoxifying enzyme for the bacteria.[75] The total synthesis of SF2768 along with the synthesis of related acyclic analogues (**66**–**68**) has also been reported (Figure 21)),[134] together with a possible binding mode (**69**) for the synthesized acyclic analogues (Figure 22).

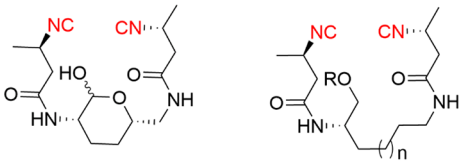

Figure 21. SF2768 and its open-ring analogues.

Hazimycin 5 and 6 (**70**) are a class of moderately active antibiotics isolated from the bacterial strain *Micromonospora echinospora*. Chemically speaking, they are tyrosine dimer analogues containing two isocyano groups. The bis-isocyano group is fundamental for the antimicrobial activity. Hazimycins have shown a broad antibiotic spectrum against *Pseudomonas*, *Candida*, *Staphylococcus*, *Sarcina*, and *Escherichia coli* (Table 7).[135,136] It is important to highlight that the subcutaneous LD_{50} for this type of molecules is 4–20 times higher than PD_{50}.[136]

Indisocin (**71**) is a highly unstable compound obtained from *Nocardia blackwellii* which showed to be strongly active against

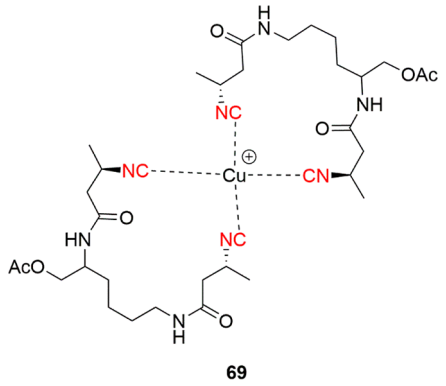

Figure 22. Proposed copper binding mode for a ring-opened analogue of SF2768. The square planar geometry was reported in the original paper, but probably it should be tetrahedral (4-coordinate d10 metal).

Table 7. Selected Antimicrobial Activity (MIC μg/mL) of Hazimycin 5 and 6 Mixture (70)

microorganism	hazimycin 5 and 6 (**70**)
Staphylococcus sp.	8
S. griseus	17.5
Klebsiella sp.	>128
Pseudomonas sp.	>128
C. albicans	17.5
S. cerevisiae	17.5
Salmonella sp.	>128

Gram + and − bacteria and fungi. Unnatural *N*-methylidisocin was also prepared and the compound maintained similar potency to the parent compound (Figure 23; Table 8).[137]

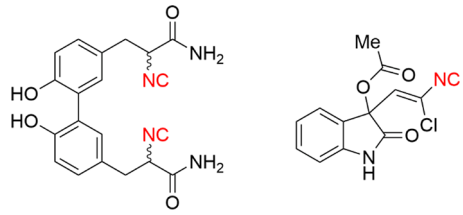

Figure 23. Hazimycin and indisocin.

The antibiotic aerocyanidin (**72**) was isolated from bacillus *Chromobacterium violaceum*. It is a fatty acid analogue,

Table 8. Selected Antimicrobial Activity (MIC μg/mL) of Indisocin and *N*-Methylindisocin

microorganism	indisocin (**71**)	*N*-methylindisocin
B. anthracis	0.2	1.56
S. aureus 209P	0.05	0.1
E. coli K-12	0.2	0.78
K. pneumoniae	0.2	0.39
P. auruginosa	25	25
S. typhi	0.78	3.12
A. niger	10	ND
C. albicans	10	ND
S. cerevisiae	2.5	ND

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containing an isocyano epoxide. This compound shows a potent activity against Gram positive bacteria, especially against *Staphylococcus aureus* (MIC < 0.05 μg/mL; Table 9).

Table 9. Selected Antimicrobial Activity (MIC μg/mL) of Aerocyanidin (72)

microorganism	aerocyanidin (72)
S. aureus	<0.05
S. faecalis	0.2
K. pneumoniae	>50
P. mirabilis	1.6
P. aeruginosa	>50
E. coli SC10909	1.6

One of its mode of actions is correlated with its ability to release cyanide anions under basic and acidic conditions (pH >8 and <4). Indeed, the increased pH activates the secondary alcohol triggering a Payne rearrangement with the formation of a pseudocyanidrin, which collapses liberating the cyanide ion (Figure 24).[138] Although its toxicity is similar to that sodium cyanide, its activity against *S. aureus* in an agar diffusion assay is larger than sodium cyanide suggesting that the release of the cyanide anion cannot be the only mechanism of action.

YM-47515 (**75**) from *Micromonospora echinospora* is a homologue of aerocyanidin displaying the same mechanism of action and spectrum of activity.[139]

Another related antibiotic is amycomicin (**76**; Figure 25), discovered by employing an approach that exploites the ability of microbial communities to produce antibiotics that are not produced by any single member. In the said experiment, *Amycolatopsis* sp. AA4 was the producing strain while *Streptomyces coelicolor* M145 was the inducing strain. Antibiotic **76** differs from aereocyanidin for the presence of a keto group on the paraffinic chain and a slightly different pattern of substitution for the epoxide. Its antibiotic activity is similar to those of aerocyanidin, being highly potent against *S. aureus* (MIC approximately 30 nM). This compound is able to inhibit the enzyme β-ketoacyl−acyl carrier protein synthases III (FabH) which is involved in fatty acid biosynthesis. The high specificity of this antibiotic for *Staphilococcus aureus* and its inefficacy against other bacteria (e.g., *Bacillus subtilis*) is ascribed to structural modifications of FabH enzyme which prevent the binding between the antibiotic and the enzyme.[71] A recent work was carried out with the goal of demonstrating the role of the epoxyisocyanide as a pro-drug, generating the epoxyketone **77** in vivo. A series of epoxyketones were therefore synthesized and evaluated for their antibacterial properties. Compound **77** was still active but with a highly reduced potency in comparison to amycomicin, highlighting that the presence of the isocyano group is fundamental for the antibiotic potency of the latter (Figure 25).[140]

The indole acryloisonitrile (**11**), originally named as B371, was isolated from *Pseudomonas* and displayed a potent antimicrobial and antifungal activity[141] (Table 10). Another indole derivative was then isolated from the actinomycete *Nocardia brasiliensis* and named brasilidine A (**79**; Figure 26). This compound showed to be cytotoxic against several tumor cell lines (e.g., CHO IC_{50} = 3.26 μg/mL; KB IC_{50} = 0.75 μg/mL; L1210 IC_{50} = 0.25 μg/mL; P388 IC_{50} = 0.44 μg/mL). It also displayed potent activity against *Aspergillus niger* and *Mycobacterium smegmatis* with MICs of 0.39 and 0.78 μg/mL, respectively. It was inactive against *Staphylococcus aureus* 209P (MIC = 100 μg/mL).[142]

Thirteen synthetic analogues of indole **11** were then prepared and biologically tested. Their antibacterial and antifungal activity was evaluated via a disc diffusion test at the concentration of 1 mg/mL against *Escherichia coli*, *Bacillus subtilis*, and the fungus *Rhizomucor miehei*. All compounds tested retained antibacterial and antifungal activity and the SAR is represented in Figure 27. A series of alkyl-2-isocyanoacrylated molecules were also evaluated. It is interesting to note the lack of aspecific toxicity of these compounds, where compounds **80** and **81** displayed 1.4 and 2.1 g/kg acute toxicity in mice respectively, while the reported MIC values for compound **82** were 1.56 μg/mL against *Staphilococcus aureus* and *Tricophyton mentagrophytes*.[143,144]

Recently the enzymes (Ambl1−3/AmbP 2/AmbP3) involved in the biosynthesis of these alkaloids have been

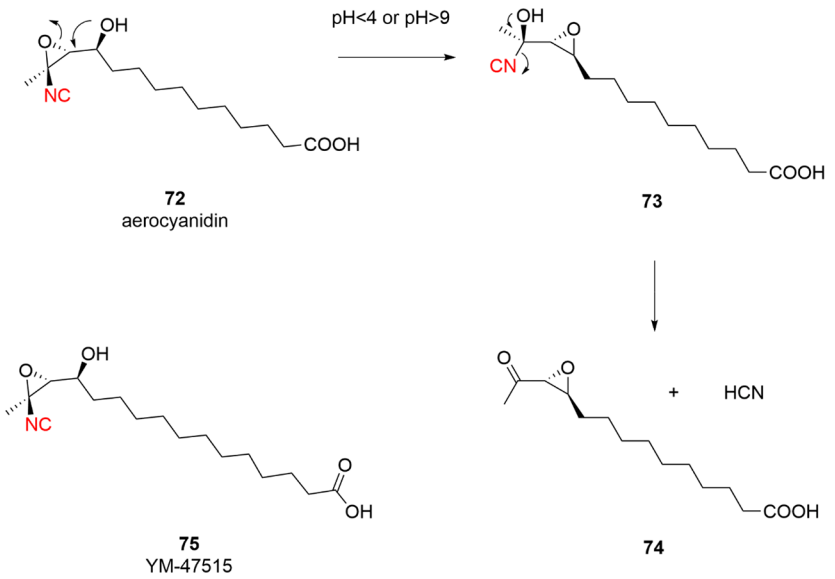

Figure 24. Mechanism of formation of HCN from aerocyanidin (**72**) and structure of YM-47515 (**75**).

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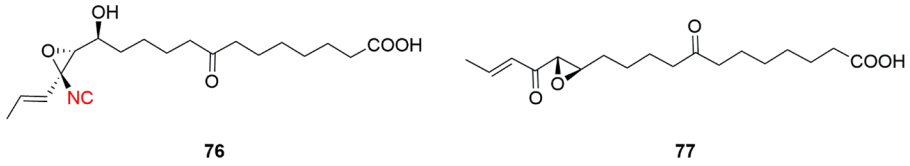
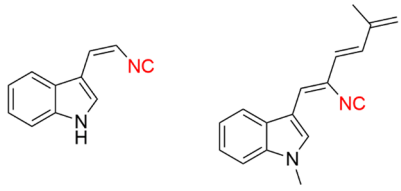


76 77

Figure 25. Amycomicin and its product of degradation.

Table 10. Selected Antimicrobial Activity (MIC μg/mL) of 11

microorganism	11
S. aureus	8
E. coli Glaxo C1343	16
P. pyocyanea	>500
Flavobacterium sp. *Glaxo C1980*	2
S. carlsbergensis	125



11 B371 **79** brasilidine A

Figure 26. B371 (**11**) and brasilidine A (**79**).

isolated[73] and their uses for preparing unnatural *cis*-indolyl vinyl isocyanides have been reported.[145]

Isocyanides are also produced by some genus of cyanobacteria. The main difference between bacteria and cyanobacteria is that the latter contain chlorophyll a, and they are phototrophic organisms. The majority of isocyanides isolated from cyanobacteria are associated with tryptophan[53,146−155] and therefore they are classified as indole alkaloids. Their biosynthetic precursors are *cis*-indolyl vinyl isocyanide and geranyl pyrophosphate, and at least three hypotheses for the biosynthetic formation of the tri- and tetracyclic cores have been made.[72,155−157]

For the sake of simplicity, we have divided them into six main groups: (a) trycyclic hapalindoles; (b) tetracyclic hapalindoles; (c) hapalindolinones; (d) tetracyclic ambiguines; (e) pentacyclic ambiguines; and (f) fisherindoles and welwitindolinones (Figure 28−33).

We collected the antimicrobial activities and the cytotoxicity against several cell lines for all of the isocyanide containing indole alkaloids reported in patent and journal literature (Table 11).[148−150,158−160]

Aside from the antibacterial activity, additional studies aiming to define the mechanism of action of some isocyanide indole alkaloids have been reported. For example, 12-epi-hapalindole E (**85**) inhibits RNA synthesis in *Bacillus subtilis*, blocking the RNA polymerase with a K_i of 1.3 mM. The low potency against RNA polymerase associated with the high activity against *Bacillus subtilis* (MIC = 3 μM) suggests that this cannot be the only mechanism of action at play for the antibiotic activity of this compound.[161,162] When 12-epi-hapalindole H was tested on zebrafish (*Danio rerio*) embryos, it showed to be teratogenic at a concentration similar to those previously observed for the antimicrobial activity.[152] In order to identify the biological mechanism responsible for the insecticidal action of hapalindoles,[147] a study on four hapalindoles [12-epi-hapalindole J (**96**), 12-epi-hapalindole C (**86**), hapalindole L (**97**), and 12-epi-hapalindole E (**85**)] was carried out. These four compounds were not cytotoxic on mammalian cells like Clone-9 hepatocyte cell line and human neuroblastoma BE(2)-M17 cancer cells at concentrations of 10 μM. They were able to block the sodium voltage dependent channels blocking the depolarization induced by veratridine with a mechanism similar to neosaxitoxin. The IC_{50} required to inhibit the veratridine-induced fluorescence was calculated for 12-epi-hapalindole C (**86**), hapalindole L (**97**), and 12-epi-

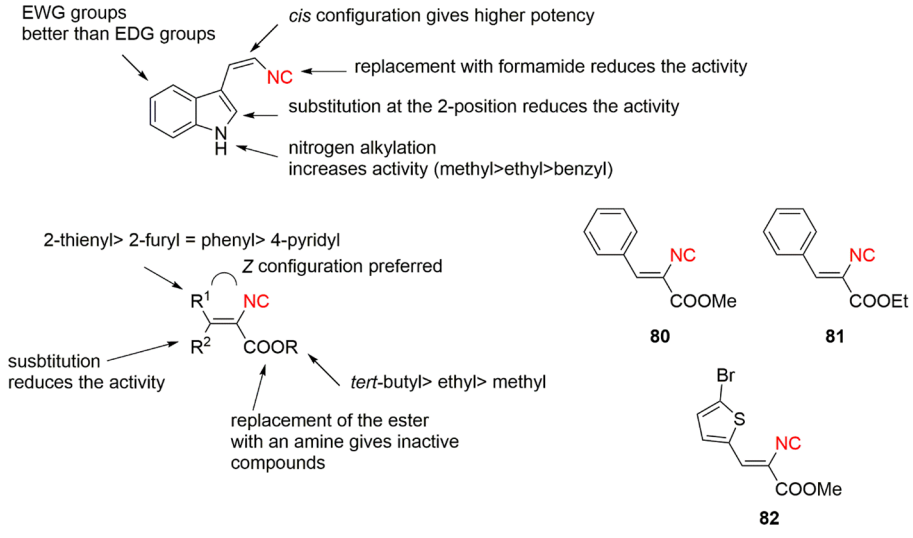


Figure 27. SAR for antibacterial isocyanides and structures of three synthetic analogues of B371 (**80−82**).

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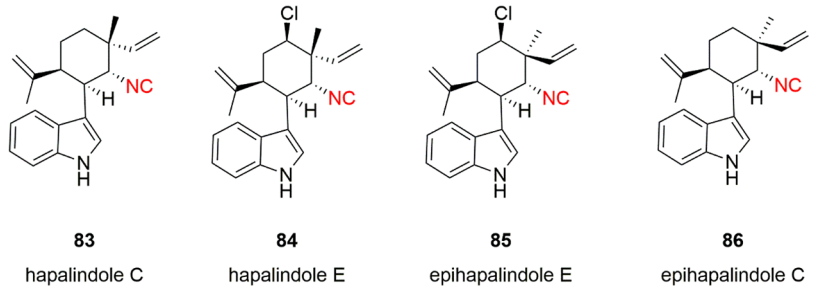

Figure 28. Tricyclic hapalindoles.

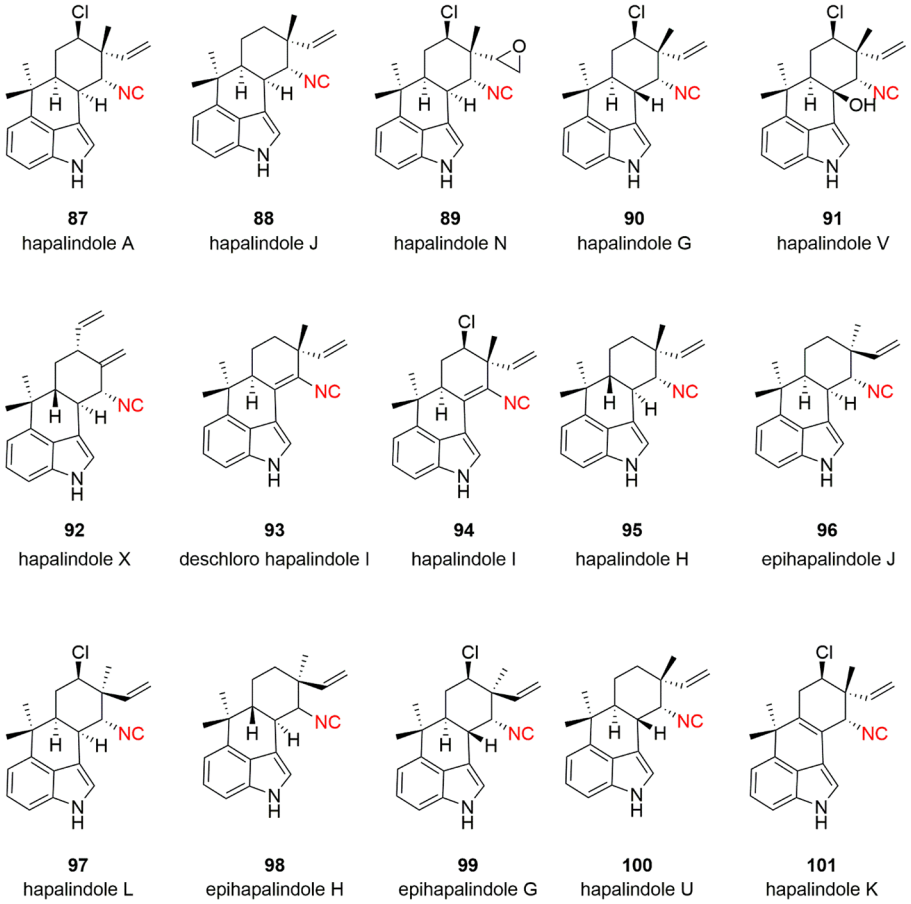

Figure 29. Tetracyclic hapalindoles.

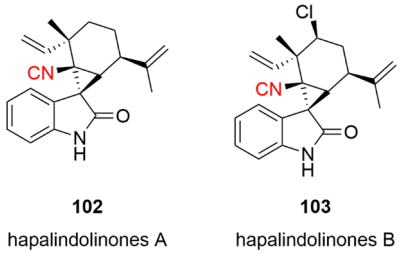

Figure 30. Hapalindolones

hapalindole E (**85**) being 4.8, 6.7, and 10.6 μM, respectively [Table 11].

Ambiguine I (**113**) was reported to be a potent inhibitor of NF-kB with an IC_{50} of 30 nM. This molecular activity is translated into its cytotoxicity against HT-29 colon cancer cell (EC_{50} of 4.35 μM) and against MCF-7 breast cancer cell line, with an EC_{50} of 1.7 μM, by blocking the cell in G1 phase.[163]

Hapalindolinone A (**102**) is an antagonist of vasopressin with a IC_{50} of 37.5 μM [Table 12].[154,164]

The 12-epi-hapalindole (**96**) was isolated from the biofilm formed by the cyanobacteria of genus *Fischerella*, and its ecological role was unveiled. Indeed, this compound was shown to be able to kill all the larvae of the dipteran *Chironomus riparius* at 26 μM in 48 h. As insect larvae are grazers of freshwater cyanobacterial biofilm, it is reasonable to imagine that the bacteria biofilm of *Fischerella* is rich in this insecticidal substance for its protection. The possibility of using specific isocyanides as novel insecticidal compounds should therefore be considered by chemists.[147]

Ambiguine D (**110**) also exerts a phytotoxic action, for example suppressing the growth of the lettuce root. At least two pathways can be responsible for its action: the inhibition

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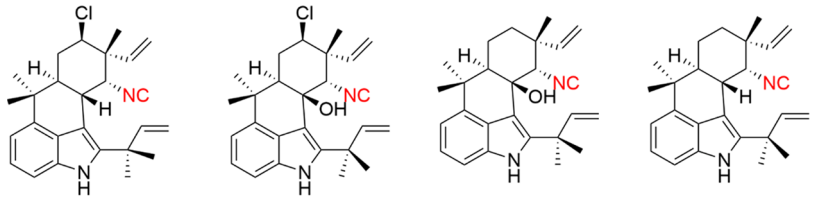

Figure 31. Tetracyclic ambiguines.

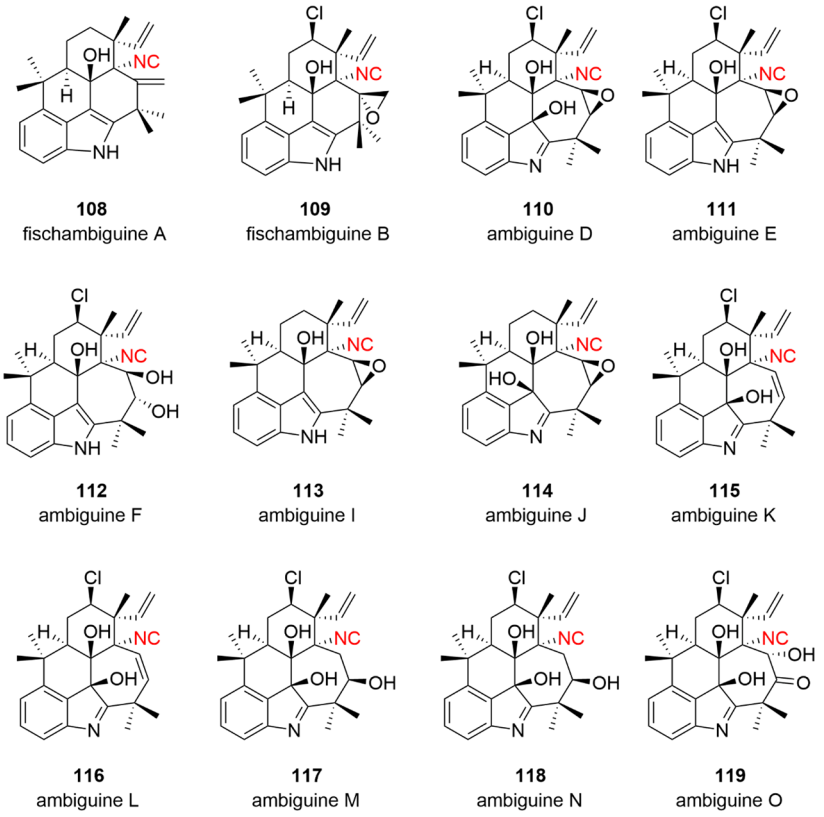

Figure 32. Pentacyclic ambiguines.

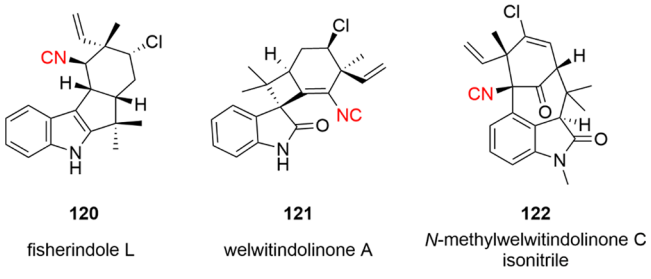

Figure 33. Fisherindoles and welwitindolinones.

of mitosis and the increased formation of reactive oxygen species [Table 13].[165]

A completely different class of compounds was isolated from cyanobacteria *Scytonema mirabile*, comprising mirabilene-A-isonitrile (**123**), mirabilene-B-isonitrile (**124**), mirabilene-C-isonitrile (**125**), mirabilene-D-isonitrile (**126**), mirabilene-E-isonitrile (**127**), and mirabilene-F-isonitrile (**128**).[166] Although no compound specific biological activity for this fascinating class of isocyanides has been reported, general cytotoxicities against LoVo and Kb cells were 5 and 1−10 μg/mL, respectively (Figure 34). A weak antimicrobial (Gram +) and antifungal activity against *Aspergillus oryzae* e *Penicillium notatum* has been shown.

3.4. Isocyanides in Marine Organisms and Their Biological Activity

The marine world is the most prolific environment for the synthesis of natural products containing the isocyano functional group. These types of compounds are found in sponges (above all of genus *Axinellida*, *Haplosclerida*, and *Halichondria*) and nudibranchs of genus *Phyllidia*. Because of their soft bodies, sponges are unable to escape from predators, and during evolution they have developed defensive mechanisms based on the production of toxic chemicals. For this reason, sponges are nowadays considered one of the most diverse sources of chemicals for researchers.[167] Marine isocyanides are associated with sponge cell membranes, and beyond defense roles, additional ones involved with the membrane functions have been postulated.[168] As already stated at the beginning of the review, nudibranchs are usually colored, shell-less mollusks

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Table 11. Selected Antimicrobial Activity of Hapalindoles and Fischerindole L (MIC μM)

compound	*M. tuberculosis*	*C. albicans*	*S. aureus*	*E. coli*	*A. baumannii*	*A. fumigatus*	vero cells IC_{50} (μM)
hapalindole A (**87**)	<0.6	1.2	3.9	8.0	>100	80	25.6
hapalindole C (**83**)	ND	0.6	16	>64	ND	ND	ND
hapalindole E (**84**)	ND	0.3	4	16	ND	ND	ND
hapalindole G (**90**)	6.8	10	>128	ND	ND	>128	ND
hapalindole H (**95**)	58.8	5.1	>128	ND	ND	>128	ND
hapalindole I (**94**)	2.0	>100	>100	>100	>100	ND	>100
hapalindole J (**88**)	4.3	0.7	8.4	>100	>100	ND	31.9
hapalindole X (**92**)	2.5	2.5	9.1	>100	>100	ND	35.2
fischerindole L (**120**)	22	1.2	6.4	>100	>100	ND	<9.2
12-epi-hapalindole E (**85**)	ND	ND	11	3	ND	ND	ND

Table 12. Selected Cytotoxic Activity of Hapalindoles (IC_{50} μM)

compound	HT-29	MCF-7	SF-268	myeloma
hapalindole A (**87**)	31.3	30.7	16.3	ND
hapalindole C (**83**)	52.6	>100	88.6	ND
hapalindole H (**95**)	10.8	16.3	10.6	ND
hapalindole I (**94**)	ND	>100	93.1	ND
hapalindole J (**88**)	28.6	43.7	16.9	ND
hapalindole X (**92**)	24.8	35.4	23.5	ND
hapalindole U (**100**)	52.6	>100	>100	ND
12-epi-hapalindole E (**85**)	ND	ND	ND	45

Table 13. Selected Antimicrobial Activity of Ambiguine and Fischambiguines (MIC μM)

compound	*M. tuberculosis*	*B. anthracis*	*S. aureus*	*C. albicans*	vero cells IC_{50} (μM)
ambiguine A (**104**)	46.7	1.0	1.8	<1.0	26.0
ambiguine B (**105**)	ND	3.7	10.9	1.7	58.6
ambiguine C (**106**)	7.0	16.1	7.4	<1.0	78.3
ambiguine D (**110**)	ND	ND	ND	1,25	ND
ambiguine E (**111**)	21.0	3.6	1.5	<0.9	42.6
ambiguine F (**112**)	61.2	ND	ND	1.25	57.9
ambiguine I (**113**)	13.1	>128	8.9	1.7	>128
ambiguine K (**115**)	6.6	7.4	4.6	<0.9	53.2
ambiguine L (**116**)	11.7	16.2	10.5	<1.0	44.6
ambiguine M (**117**)	7.5	28.5	4.7	1.1	79.8
ambiguine N (**118**)	27.1	30.9	5.5	<1.0	118.4
ambiguine O (**119**)	ND	13.8	ND	ND	80.7
fishambiguine A (**108**)	>100	ND	82.3	15.3	ND
fishambiguine B (**109**)	2	28.7	19.4	15.3	>128

and do not synthesize isocyanides on their own, but they obtain them from dietary sources and repurpose them as protective agents against predators.

One of the first isocyanides to be isolated in nudibranchs was 9-isocyanopupukeanane (**129**) from *Phyllidia varicosa*,[169] later found to also occur in the sponge of genus *Ciocalypta*. In the years to follow, the C-9 epimer of 9-isocyanopupukeanane (**130**), 2-isocyanopupukeanane (**131**),[59] 9-isocyanoneopupukeanane (**132**),[62] and 2-isocyanoallopupukeanane (**133**)[52] was isolated (Figure 35).

The anecdote reported by the late Prof. Paul J. Scheuer renders a clear picture for the potent ichthyotoxicity of 9-isocyanopupukeanane (**129**). Prof. Scheuer's interest in chemical marine ecology began when a friend added the nudibranch *Phyllidia varicosa* to his aquarium. To their surprise, as time went by they witnessed the death of all the fish and shrimp in the aquarium, while the nudibranchs appeared alive and well![170] Shortly after, 9-isocyanopupukeanane (**129**) was isolated from the skin secretion of the nudibranch. This compound is more ichthyotoxic than its epimer with an LD_{50} of 1.0 μg/mL versus 2.0 μg/mL.[171] 2-Isocyanoallopupukeanane (**131**) showed an ichthyotoxicity of 10 μg/mL (LC_{50}) against *Oryzias latipes*.[52] In other cases, the isocyanide is not released but concentrated on the skin behaving as a feeding deterrent, protecting the nudibranchs from predators. Indeed, no predators for *Phyllidia varicosa* are known. The lipidic isonitrile compound actisonitrile (**134**) was isolated from the nudibranch *Actinocyckus papillatus*, and its total synthesis was reported, including its enantiomer. Cytotoxicity was evaluated for the two enantiomers. Both showcased a similar biological profile. In detail, their biological activity was tested against tumor C6 rat glioma cells (IC_{50} = 41 μM), HeLa human cervical cancer cells (IC_{50} = 35 μM), nontumor H9c2 rat cardiac myoblasts (IC_{50} = 23 μM) and 3T3-L1 murine fibroblasts (IC_{50} = 73 μM).[172] Additional isocyanides have been isolated from other *Phyllidia*; however, those molecules had been already isolated from sponges. It is important to note that many of these isocyanides are highly volatile oils, a property complicating their isolation from natural sources.[173]

The very first marine isocyanide was discovered by a group of talented Neapolitan researchers lead by Professor Ernesto Fattorusso in 1973 from the Mediterranean sponge *Axinella cannabina*. The compound was named axisonitrile-1 (**135**).[174] This compound was shown to be toxic against marine fish *Chromis chromis* and *Carassius carassius* at a concentration of 8 ppm. One year later, still in Naples, the isocyanide achantellin-1 (**136**) was isolated from the same sponge[175] (Figure 36). From this moment on, numerous isocyanides were isolated and characterized from said sponge and many others (*Halichondria, Pseudaxinella, Acanthella, Bubaris, Cribochalina, Cymbastela, Amphimedon, Ciocalypta, Geodia, Phakellia,* and *Svenzea*) and evaluated for their biological activity whenever possible, with an emphasis on their cytotoxicity against cancer cell lines and their antimalarial activity. For the sake of simplicity, we are grouping those isolated isocyanides as shown

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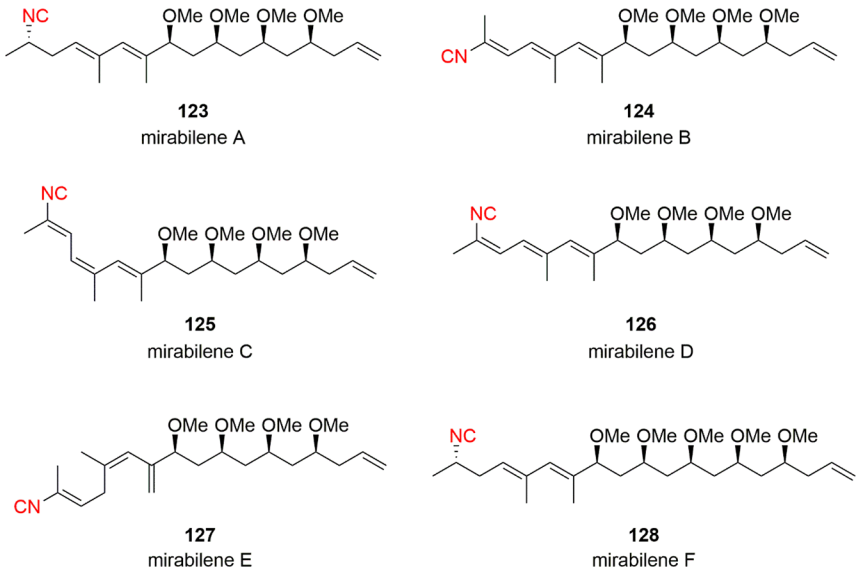

Figure 34. Mirabilene isocyanides.

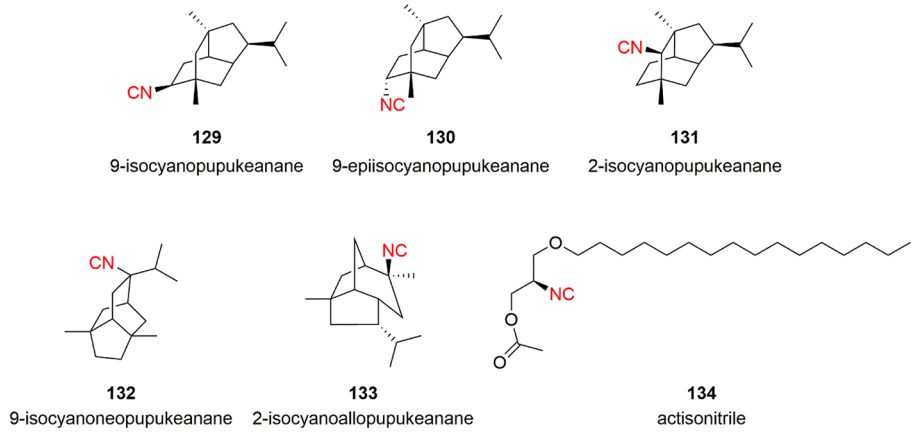

Figure 35. Isocyanides obtained from nudibranchs.

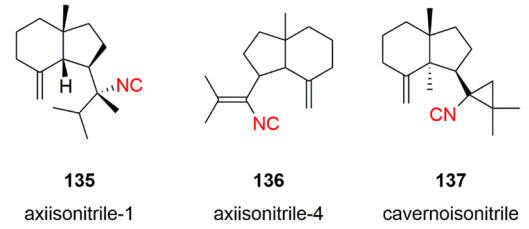

Figure 36. Hydrindane derivatives.

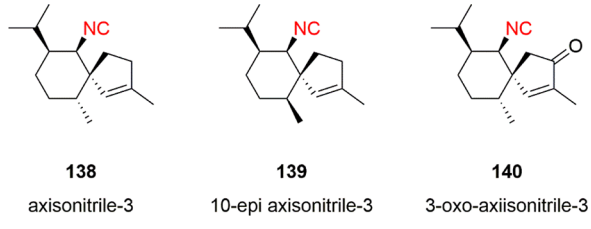

Figure 37. Spiro[4.5]decane derivatives.

in the figures, and for each represented group we report their published biological activities.[61,62,176−206]

Axisonitrile-3 (**138**) displayed a strong activity against HepG2 cell line with an IC_{50} of 1.3 μM, 2.44 μM on A549, 0.7 μM on P388 cells, and KB and LoVo cells with a cytotoxicity <1 μL/mL, while its formamide analogue was inactive (Figure 37). On the other hand, this isocyanide was not cytotoxic against other cancer cell lines (e.g., HeLa, HuCCA-1, A549, and MDA-MB-231).[207−210] The same compound was active against *Plasmodium falciparum* with an IC_{50} of 142 and 16.5 ng/mL on D6 and W2 strains, respectively (SI = 1212). No cytotoxicity on KB cell at a concentration >20 μM was reported. The compound is also active against *Mycobacterium tuberculosis* with an MIC of 2 μg/mL.[211] When the isocyano group was replaced with an isothiocyanate, the activity dropped by a factor of 500.[212] The 10-epiaxisonitrile-3 (**139**) was isolated from the marine sponge *Geodia exigua*. This molecule was able to inhibit the fertilization of sea urchin and starfish gamete at a concentration of 0.4 μg/mL, due to its ability to immobilize the sperm, by inhibition of the phosphocreatine shuttle. Its formamido analogue was completely inactive. It is interesting to note that in this work, the Authors synthesized simpler isocyanides like benzyl isocyanide, cyclohexyl isocyanide, and 6-isocyanospiro[4.5]decane. All of them were devoid of activity against the mobility of sperm.[213]

Haliconadin C (**144**) is an eudesmane sesquiterpenoid containing the isocyanide moiety, isolated from the marine

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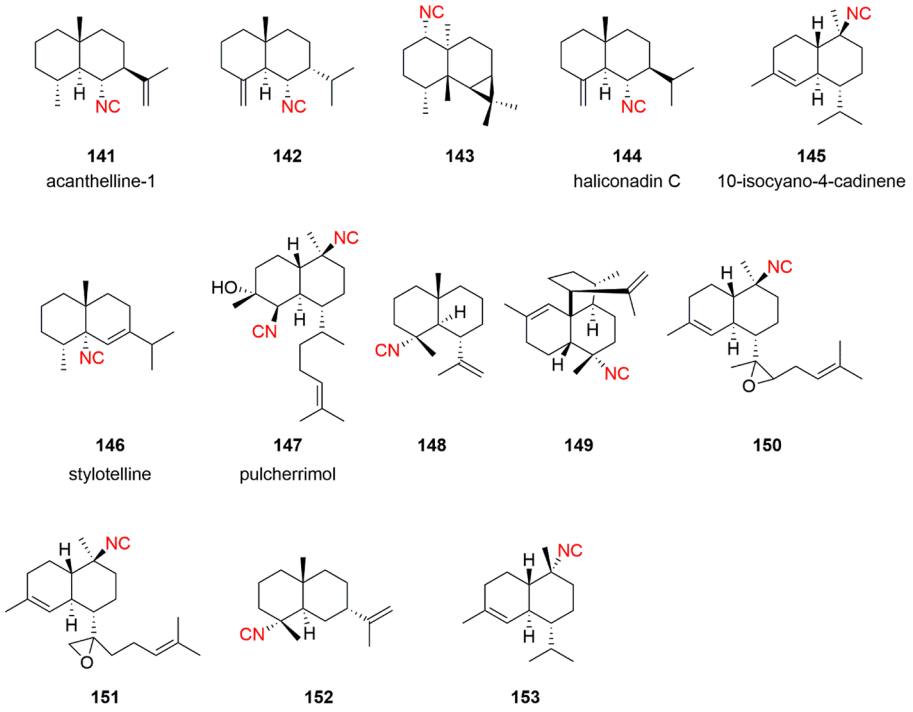

Figure 38. Eudesmane and *trans*-decalin derivatives.

sponge of genus *Halichondria* (Figure 38). This compound has the strongest antifungal activity against *Micrococcus luteus* (MIC 0.52 μg/mL) and *Cryptococcus neoformas* (MIC = 0.0625 μg/mL) while being inactive as antibacterial.[214] It was also isolated in complex with copper(I) **154** (Figure 39). This complex showed a reduced antimicrobial activity when compared to the copper-free halicondin C against *Micrococcus luteus*, and *Cryptococcus neoformans*, with MICs of 4 and 16 μg/mL, respectively.[215] Compound **148** was cytotoxic against P388 cells with an IC_{50} of 8.3 μg/mL.[216]

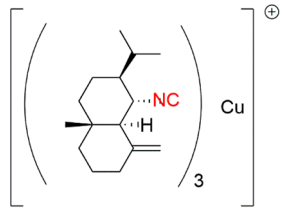

Figure 39. Copper complex of halicondin C.

10-Isocyano-4-amorphene (**155**; Figure 40) was isolated from nudibranchs of the family *Phyllidiidae* along with 10-isocyano-4-cadinene (**145**). These compounds have a potent antifouling activity against the larvae of the barnacle *Balanus amphitrite* (EC_{50} = 0.7 and 0.14 μg/mL, respectively).[217] The total synthesis of **145** (+), its enantiomer (−), and the diasteroisomers have been reported along with their biological activity. The single enantiomer (+) was the most potent, with an EC_{50} of 0.06 μg/mL, the enantiomer (−) had an EC_{50} of 0.08 μg/mL while both the diastereoisomers were less active, with an EC_{50} between 0.21 and 0.40 μg/mL.[218] The antimalarial activity of compounds **145** is 705 nM (EC_{50}) against *Plasmodium falciparum* 3D7, 247 nM against Dd2 strain.[219]

The hydroxyl functionalized isocyanosesquiterpene **158** was isolated from the sponge *Phyllidia pustolosa*. This compound showed antifouling activity against cypris larvae of the barnacle *Balanus amphitrite* with an EC_{50} of 0.17 μg/mL and a lethality below 5% at the same concentration (Figure 40).[220] The isocyanide sesquiterpenes halichon C (**156**) and 4-epi-halichon C (**159**) were isolated from the sponge *Halichondria*. The compounds were evaluated for their cytotoxicity against a panel of cancer cell lines resulting in poor levels of activity.[221] We are perplexed regarding the presence of the hydroperoxide moiety in **156** and **159**.

1-Isocyanoaromadendrane (**160**) proved toxic for the fish *Lebistes retisulatus* with an LD_{50} of 30 mg/L. Compound **165** was the first isocyano derivate with a guai-6-ene skeleton and displayed high levels of cytotoxicity: ED_{50} = 0.19 μg/mL (L-1210) and 0.27 μg/mL (HeLa; Figure 41).[222] Regarding

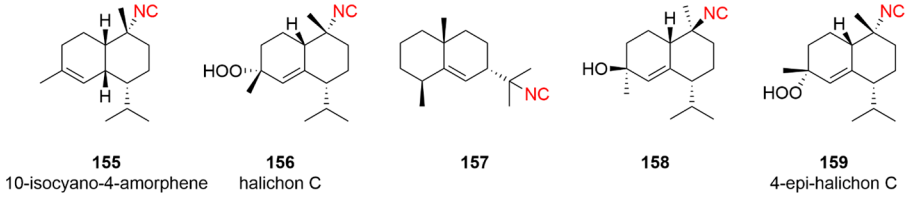

Figure 40. Eudesmane and *cis*-decalin derivatives.

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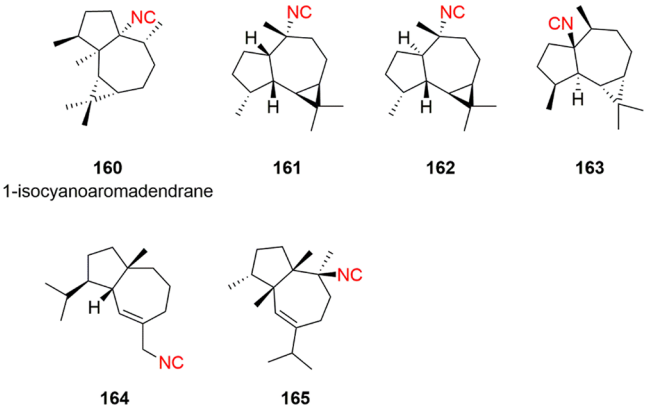

Figure 41. Aromadendrane derivatives.

compound **164**, the presence of a primary isocyanide is quite strange in light of the biosynthesis of marine isocyanides. Indeed, they are only formed via cyanide trapping of stabilized secondary or tertiary alkyl carbocations.

More than 50 isocyanide functionalized molecules, named kalihinols, have been isolated from the sponge of genus *Acanthella*. It is possible to divide the kalihinols into two main groups: (a) kalihinols with a *trans*-decalin and a tetrahydropyranyl ring (Figure 42) and (b) kalihinols with a *trans*-decalin and a tetrahydrofuranyl group (Figure 43). It is interesting to highlight that only few of them have been tested for their biological activity and generally only as antimalarial compounds. The lack of biological data around the kalihinols is a consequence of their scarce availability. For instance, the yield of kalihinol A **166** from the tissues of *Acanthella cavernosa* is 0.0016% of the wet weight of the sponge.[223]

Kalihinols were shown to inhibit the settlement of cyprid larvae of the barnacle *Balanus amphitrite*. For example, Kalihinol A (**166**) displayed potent antifouling activity with an EC_{50} = 0.087 μg/mL[224,225] against larvae of *Balanus amphitrite*. Kalihinol E (**167**) inhibits larval settlement and metamorphosis with an EC_{50} of approximately 0.5 μg/mL.[196]

Kalihinols showed a marked antimalarial activity (see Table 14) as well as antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and antimycotic against *Candida albicans*, while being inactive against *Escherichia coli*.[183,184] Although at the very high concentration of 115 μg/mL, Kalihinol F (**176**) was shown to be a topoisomerase I inhibitor, preventing the chromosome separation in fertilized starfish *Asterina pectinifera*.[226] Kalihinols A (**166**), X (**168**), and Z (**170**) were also very active as anthelmintic against *Nippostrongylus brasiliens* at 50 μg/mL, while isokalihinol F (**180**) was inactive.

Kalihinol A (**166**) is the most potent antimalarial agent for this class of compounds. Simplified analogues of kalihinol A have been prepared and evaluated for their biological activity. Many of these derivatives maintain their antimalarial activity against *Plasmodium falciparum* Dd2 strain even when the THP ring of kalihinol A was removed, as well as when configuration of tertiary isocyanide was reversed. In detail, compound **193** with an IC_{50} of 1.6 nM on Dd2 strain maintained the same potency of kalihinol A, while its monoisocyanide analogue **194** lost potency (Figure 44). While a precise pharmacophore model for kalihinols is not clear, the role played by the isocyano group appear pivotal: when it was replaced by a cyano, an azide, a formamide, or an isothiocyanate group, the compounds were inactive. The structure−activity relationships that emerged from this study highlight the fact that the extreme structural complexity of the kalihinols is probably not essential in order to achieve high levels of antimalaria activity (see also paragraph 6.2). It would also appear that the configuration of the C-10 isocyano group does not play a significant role. Interestingly, racemic **192** was evaluated for its microsomal metabolic stability in human and rat liver microsomes, resulting in a half-life of 142 min in human and 87 min in rat, respectively.[219]

Kalihinol A (**166**) was also reported to exert inhibition activity against COX2 with an IC_{50} of 1.07 μM.[204]

The antibacterial and cytotoxic activity for several kalihinols is reported in Tables 15 and 16.

It was shown that a possible mechanism for the antimicrobial action of kalihinols is the inhibition of the

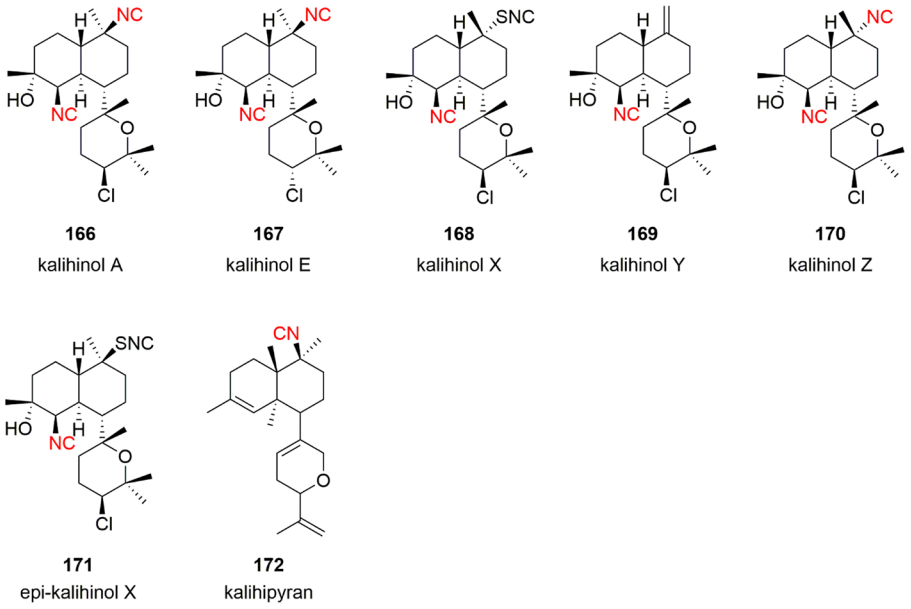

Figure 42. Kalihinols with a *trans*-decalin and a tetrahydropyranyl ring.

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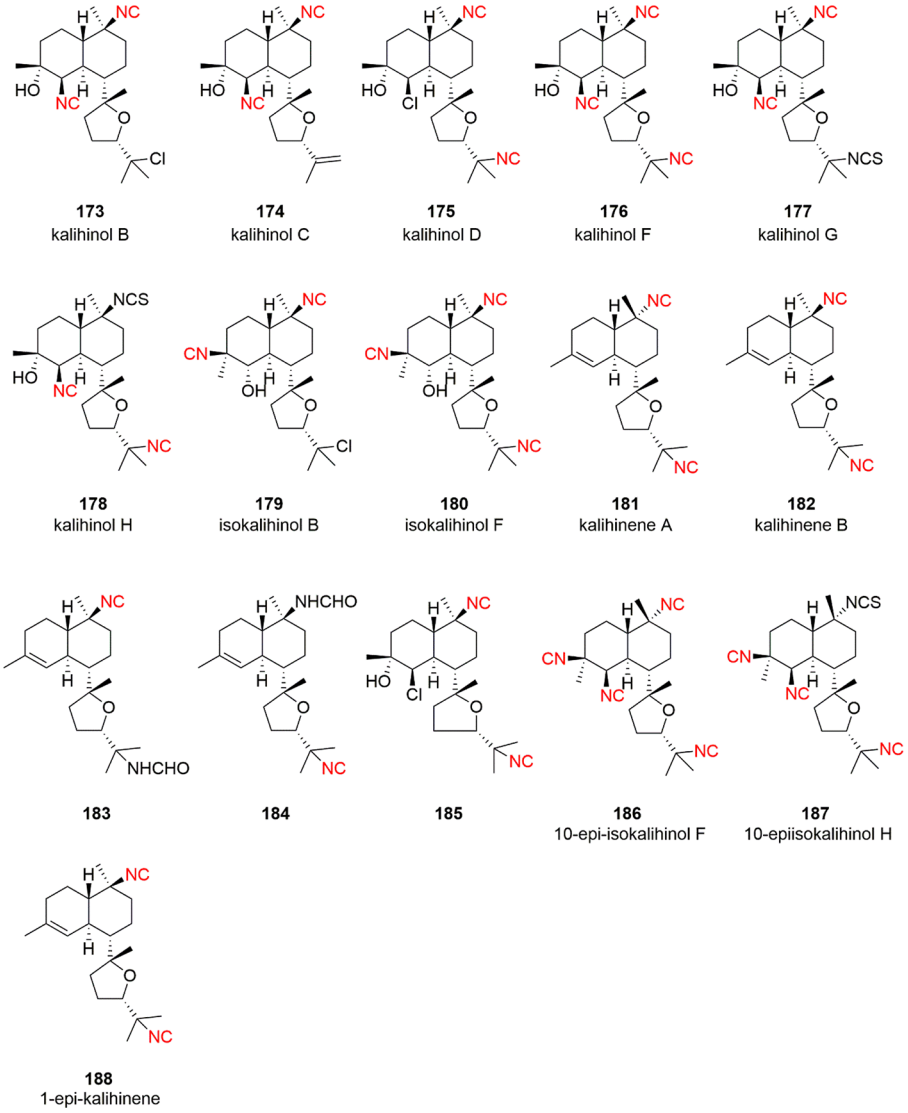

Figure 43. Kalihinols with a *trans*-decalin and a tetrahydrofuranyl group.

Table 14. Antimalarial Activities of Kalihinols[179]

compound	*P. falciparum* FCR3 (EC_{50}) (nM)	FM3A EC_{50} (nM)	selectivity index
kalihinol A (**166**)	1.2	380	317
kalihinene A (**181**)	10	37	4
kalihinol B (**182**)	4.6 (on Dd2 strain) 8.4 (on 3D7 strain)	ND	ND

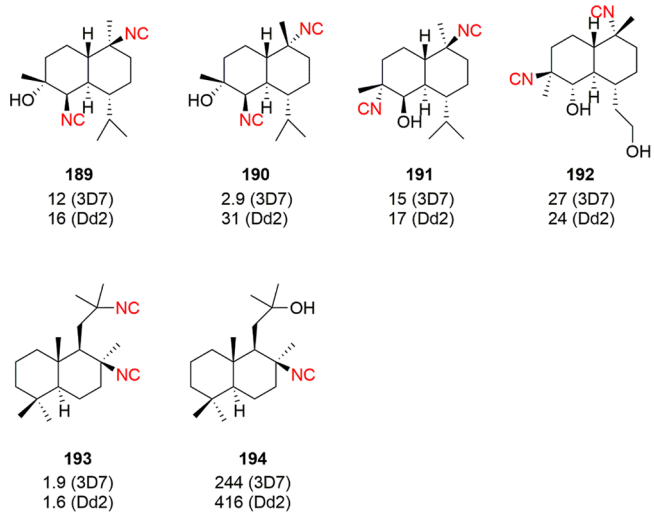

Figure 44. Antiplasmodial assay IC_{50} (nM).

bacterial folate biosynthesis. While kalihinol A (**166**) was poorly active, kalihinol Y (**169**) and kalihinol X (**168**) were the most potent compounds with an MIC of 1.56 μg/mL. Rescue assay using folic acid did not revert the action of kalihinols, pointing out that this class of compounds is endowed with more than one antibacterial mechanism of action.[229]

The ability of kalihinol F (**176**) to chelate copper has been studied in detail. Kalihinol F interacts with Cu (II) and this was demonstrated via NMR by exploiting the paramagnetic nature of copper, broadening the width of the peaks of its organic ligand. Furthermore, it was demonstrated how the kalihinol F complexing ability can reverse embryo defects caused by high copper concentration. At the concentration of 5 μM/mL, kalihinol F was able to prevent the harmful effect of copper excess (chloride 5 μM) in the embryos of zebrafish.

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Table 15. Antibacterial and Antifungal Activity of Kalihinols (MIC μg/mL)

compound	*B. subtilis* (PY79)	*T. rubrum*	*C. albicans*
kalihinol A (**166**)	50	ND	ND
kalihinol X (**168**)	1.56	ND	ND
kalihinol Y (**169**)	1.56	ND	ND
kalihinol F (**176**)	12.5	ND	ND
kalihinol G (**177**)	3.12	ND	ND
kalihinene A (**181**)	6.25	ND	ND
183	ND	8	ND
184	ND	4	8

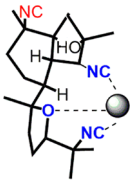
Similar results were obtained when hepatocarcinoma cells (HepG2) were exposed to high concentration of copper. In this case, it was possible to verify the upregulation of MT1B, MT1E, MT1F, MT2A, HMOX, HSPA1B and HSPCA proteins and the downregulation of COMMD1 and COMMD2. Furthermore, it was shown that kalihinol F is a weaker binder for copper than penicillamine, but it should display a better plasmatic membrane penetration in virtue of its higher hydrophobicity. An X-ray supported model for the kalihinol F copper chelation has been proposed (Figure 45). In these X-ray crystallographic structures, the two-isocyanide functional groups are parallel to each other and at a distance of 4.1 Å.[230]

3-isocyanotheonellin (**196**) was mildly cytotoxic against several cancer cell lines [GI_{50} = 27 (MDA-MB-231), 36.4 (A549), and 33.4 μM (HT-29)][231] furthermore it showed a weak virucidal activity against the A59 coronavirus in mouse liver cells. Interestingly, in another publication different cytotoxities were reported for the same compound (e.g., IC_{50} = 8.6 μM (A549), 3.35 μM (HT-29).[232] The isocyanide **198** from sponge *Ciocalypta* was endowed with activity against *Bacillus subtilis*.[233] Both **196** and **198** were cytotoxic against SNU-398 with an IC_{50} of 0.50 μM.[232]

Two novel isocyanides (**197** and **199**) were identified from the sponge *Axynyssa isabela*. Cytotoxic evaluation on three cancer cell lines pointed to compound **196** as being weakly cytotoxic, with GI_{50}'s of 27 (MDA-MB-231), 36.4 (A549), and 33.4 μM (HT-29; Figure 46).[231]

Pustolosaisonitrile-1, -2, and -3 (**202**−**204**) were isolated from nudibranch *Phyllidiella pustolosa*. Pustolosaisonitrile-1 (**202**) has an activity against *Plasmodium falciparum* 3D7 of 1.08 μM.[234]

Compound **220** (Figure 47) was evaluated for its activity against *Mycobacterium tuberculosis*. This compound has an MIC of 8 μg/mL and a cytotoxicity on Vero cell of 19 μg/mL. Interestingly, when the isocyano group was replaced with an amine the antimicrobial activity was maintained (MIC 6 μg/mL) but the compound showed no more cytotoxicity on Vero cells (IC_{50} < 128 μg/mL).[235] Compounds **221** and **223** were weak cytotoxic with an IC_{50} of 20.0 and11.2 μM respectively on HeLa cells.[236]



195

Figure 45. Copper complex with kalihinol F (**176**).

Some isocyanide amphilectanes are potent anti-inflammatory agents. Compound **215** (DINCA, 8,15-diisocyano-11(20)-amphilectene) inhibits the synthesis of TXB_2 by a cyclooxygenase-dependent mechanism with an IC_{50} of 0.20 μM on rat microglia. Interestingly, this compound is not cytotoxic with a LD_{50} > 10 μM. An initial SAR study demonstrated the pivotal role played by the two isocyano groups as well as the importance of the diterpenoid skeleton. This compound could be a lead candidate for the generation of novel anti-inflammatory agents which reduced the overproduction of TXB_2 in neuroinflammatory disorders.[237] This ability is reported for other compounds of these class, along with an antiproliferative activity.[238] Compound **205** also showed an antiproliferative effect against lymphocytes T and B stimulated by allogenic feeders or phytohemeagglutin.[239]

Antitubercular and antimalarial activity of some amphilectanes is reported in Tables 17 and 18.

Compound **215** has an antiplasmodial activity against *Plasmodium falciparum* Dd2 strain of 3.1 nM and 3D7 strain of 1.2 nM with SIs of 32 and 83, respectively.[242] The same compound was isolated from nudibranch *Phyllidia coelestis*. It displayed antifeeding properties against the shrimp *Palaemon elegans* with a minimum dose effect of 2.0 mg/mL. Interestingly, it seems that in the isocyano groups in DINCA are not of great importance for antimalarial activity. Indeed, when both of them were replaced with isoselenocyanate moiety the resulting compound maintain potent antimalarial activity [IC_{50} = 6.6 (Dd2; SI = 7356) and 2.5 nM (3D7; SI = 19)].

The same isoselenocyanate compound proved to be active against *Mycobacterium tuberculosis* with an MIC of 3.9 μM respect to 9.8 μM for DINCA (**215**).[242,243]

Compound **215** showed cytotoxitities against A549, HT-29, and P388 of 13.0, 1.2, and 0.7 μg/mL, respectively.[244]

Diisocyanoadociane (DICA) **228** showed to be cytotoxic against the following cancer cell lines: A549 (EC_{50} = 13.0 μM) HT-29 (1.2 μM), and P388 (0.7 μM). It also possesses an antimalarial activity against *Plasmodium falciparum*, with IC_{50}'s of 14 (SI = 1000) and 13.2 nM (SI = 1100) against D6 and W2, respectively.[245] **225** was moderately active against *Plasmodium falciparum* strains with IC_{50}'s of 0.2 (FCR3F86), 0.6 (W2), and 0.5 μg/mL (D6) and with a cytotoxicity against KB cell >5 μg/mL.[185] **228** showed to have an antitubercular activity of 8 μg/mL (Figure 48).[246]

Table 16. Cytotoxicity IC_{50} μM[208,227,228]

compound	HCT116	A549	HeLa	QCY-7701	MDA-MB-231	P388 (μg/mL)
epi-kalihinol X (**171**)	ND	9.30	ND	ND	ND	ND
isokalihinol B (**179**)	ND	ND	ND	ND	ND	0.8
183	>50	17.53	14.74	16.39	>50	ND
184	>50	6.98	13.30	14.53	6.84	ND

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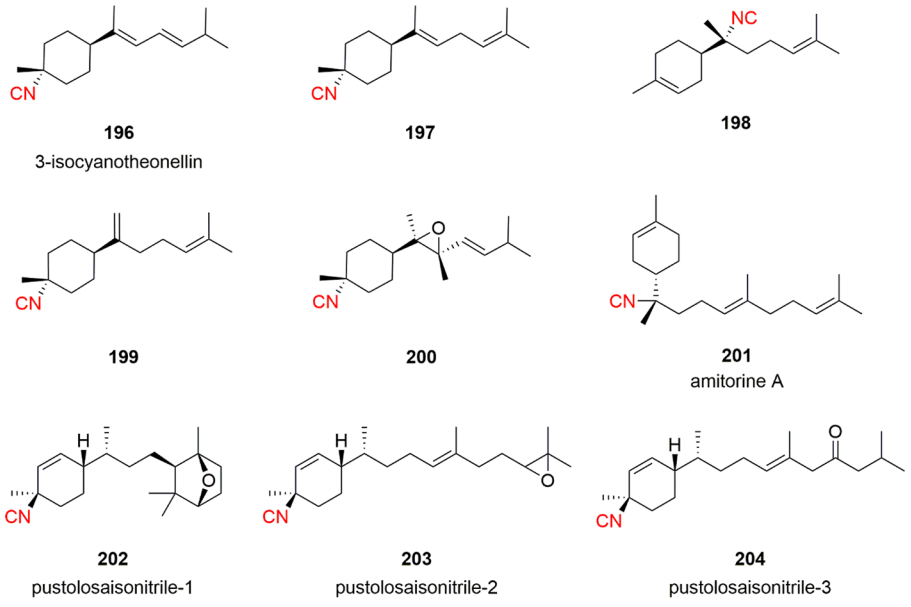

Figure 46. Bisabolane derivatives.

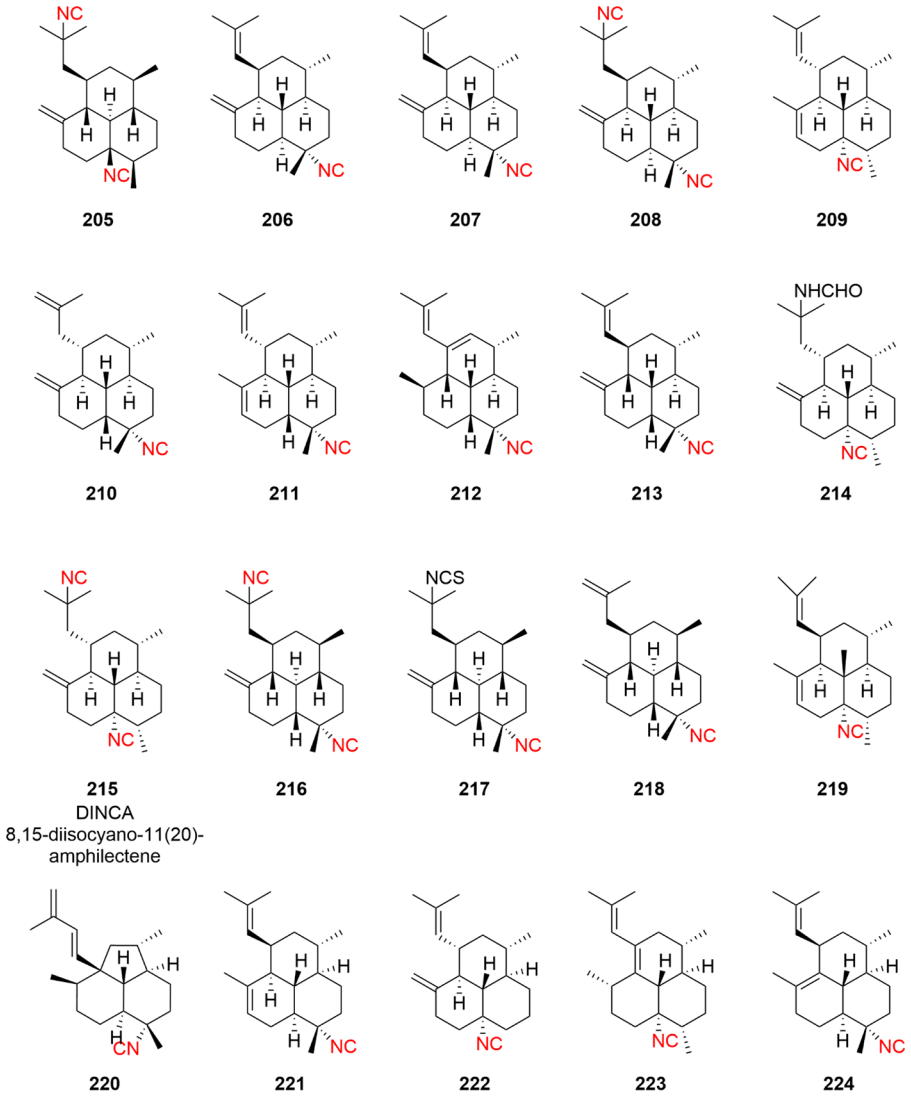

Figure 47. Amphilectane derivatives.

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Table 17. Antitubercular Activity MIC μg/mL and Cytotoxicity $IC_{50}$$\mu$g/mL of Some Amphilectane Isocyanides[240]

compound	*M. tuberculosis*	*M. avium*	KB cell
206	8	32	3.2
217	4	ND	>20
224	4	ND	15.2

Table 18. Antimalarial Activities of Amphilectanes Derivatives (IC_{50} ng/mL)[241]

compound	*P. falciparum* D6	*P. falciparum* W2	KB cell
206	14.1	9.3	3200
210	520	242	>20 000
217	470	109	>20 000
220	90	29.7	19 100
224	58.5	25.6	15 200

The epi-isomer at C-20 of DICA was prepared and evaluated for its antimalarial activity. It maintained activity with a little reduction of potency being 78 nM on Dd2 strain and 51 nM on 3D7 strain with respect of values of 15 and 2 nM for DICA.[247] These result highlights the importance of the isocyanide at the 7 position being equatorial, while the isocyano group at the 20 position is not directly involved in the interaction. Other activity of adociane derivatives as antimalarial compounds are reported in Table 19.

Compound **231** was antibacterial with an activity against *Staphylococcus aureus* and *Bacillus subtilis* of 5 μg/disk.[191]

Adociane **225** and the two already known amphilectanes **215** and **205** were isolated from *Pseudoaxinella flava*. Their activity was assayed on human cancer lines, displaying activity against PC3 cancer line with IC_{50}'s of 7 for **225**, 2.1 for **215**, and 3 μM for **205**. Similar results were shown for LoVo colon cancer cells while higher cytotoxic values were shown for U373 glioblastoma cells (10−25 μM), Hs683 (4−50 μM), SK-MEL-28 (6−32 μM), and A549 NSCLC (16−42 μM). Although the biological target of these compounds is still unknown, it was shown how compound **225** induces marked vacuolization leading to cell death. These four compounds seemed to respond well against both apoptosis sensitive cancer cell lines and cancer cell lines resistant to pro-apoptotic stimuli.[248]

Table 19. Antimalarial Activities of Amphilectanes Derivatives (IC_{50} ng/mL)[241]

compound	*P. falciparum* D6	*P. falciparum*W2	KB cell
228	4.7	4.3	4700
229	62.5	19.5	18 200
220	90	29.7	19 100

The biological activity of other marine isocyanides was further evaluated against three *Plasmodium falciparum* strains (Table 20). The antibacterial, antifungal and antialgae properties are also represented.

A possible mode of action as antimalarial for some of the isocyanides discussed above have been investigated without reaching a definitive conclusion. In 2001, it has been suggested that ferriprotoporphyrin IX was the candidate receptor.[249] In particular, it was suggested that diisocyanoadociane (**228**) and axiisonitrile-3 (**138**) could form a coordination complex between the Fe(II) of heme and the isocyano group. For instance, it was shown that axiisonitrile-3 and diisocyanoadociane were able to modify the UV Soret peak for the heme, without interacting with heme of oxyhemoglobin, probably due to steric reasons. ESI-MS studies showed a stochiometric ratio of 1:1 between heme and axiisonitrile-3 and of 2:1 between heme and diisocyanoadociane.[250] The interaction of the isocyano group with the iron of heme should inhibit the formation of the highly insoluble brown crystals of β-hematin (hemozoin). Formation of hemozoin is indeed the classic method of heme-detoxification used by the *Plasmodium* to avoid the toxicity associated with the free heme. Unfortunately, the authors did not use other hydrophobic compounds devoid of the isocyano group in order to demonstrated whether they were still able to disrupt or not the heme crystallization in vivo. In their own words they "have empirically proven that inhibition of heme polymerization is in fact the reason for the potent antiplasmodial activity". In 2015, the remarkable ability of DICA to completely inhibit the β-hematin crystallization (IC_{50} of 13 nM) was demonstrated by using the Egan's β-hematin inhibition assay. Other isocyanides maintain this ability without completely inhibiting the β-hematin crystallization (e.g., for example compound **206** inhibits at 86% the β-hematin crystallization with an IC_{50} of 31 nM).[251] When ab initio molecular modeling studies were performed, it was showed that the previously suggested

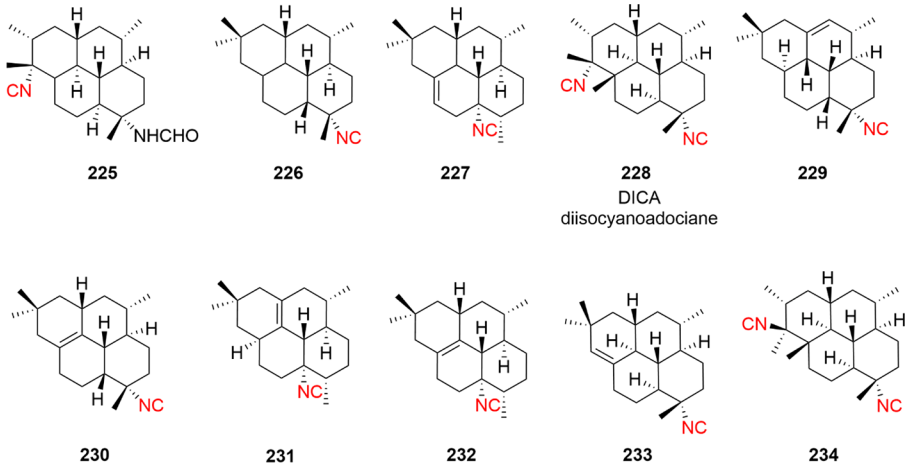

Figure 48. Adociane derivatives.

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Table 20. MIC (nM) for the Antimalarial Activity, against FCR3F85, D6 and W2 Strains and for KB Citotoxicity[a]

Cpd	FCR3F85	D6	W2	KB cytotox.	*E. coli*	*B. megaterium*	*M. microspora*	*C. fusca*
228	ND	4.7	4.3	4700	1	3	4	2
233	ND	74.1	23.8	14 500	inactive	1	6	3
206	ND	14.1	9.3	3200	inactive	2	10	3
224	ND	58.5	25.6	15 200	inactive	2	12	3
225	200	800	700	>20 000	inactive	2	5	inactive
138	ND	142	165	>200	1	2	2	inactive

[a]IC_{50} (μg/mL) for antibacterial and antifungal activities. Antialgal activity at a concentration of 50 μg/disk (inhibition was measured in mm from edge of disk)..

interaction between the isocyano group and the iron of heme was not stable. In this study, the molecules appear rather oriented by displaying the isocyano group away from the porphyrin ring, while the hydrophobic terpene scaffold is engaged in ring stacking interactions (a mixture of van der Waals and hydrophobic interactions) with the heme group (Figure 49). Even for this paper, we recommend prudence for the same reasons listed above.

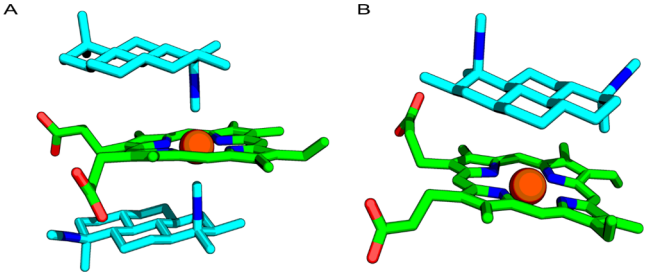

Figure 49. To be or not to be. Different hypotheses for the isocyanoterpenes binding to heme.

In this regard, the Shenvi group has synthesized and then tested amphilectanes **206** and **228** and simple isocyanides **235**, **236**, and **237** not only against the *Plasmodium* active during the blood stage, but also against the liver schizont of *Plasmodium* (Figure 50). These schizonts do not use

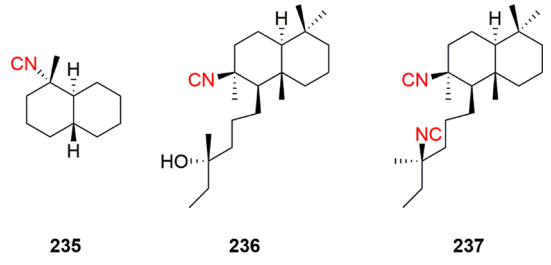

Figure 50. Simplified analogues synthesized by Shenvi's lab.

hemoglobin as a nutrient and therefore do not require the detoxification of the heme group. Interestingly, all the isocyanides were active both on blood and liver stage plasmodium, although at higher concentration (Table 21).[252] The antimalarial activity of these isocyanides against liver schizonts undermine the hypothesis of heme biocrystallization inhibition. In our opinion the true mechanism of action of the potent antimalarial isocyanides lies dormant waiting for being discovered.

Three isocyanides named monamphilectine A (**238**), B (**239**), and C (**240**), belonging to the amphilectane-type, were

Table 21. Activity against *P. falciparum* (IC_{50} nM)

compound	asexual blood-stage *P. falciparum*	liver stage *P.berghei*	HepG2
206	992	932	27 960
228	16	1296	24 130
235	>50 000	104 800	>50 000
236	150	221	1546
237	9	1546	27 960

isolated from the *Svenzea flava* sponge. Interestingly, they have a side chain with a beta-lactam ring (Figure 51).

Compound **238** was active on *Plasmodium falciparum* W2 with an IC_{50} of 0.6 μM, while **239** and **240** displayed an antiplasmodial activity against *Plasmodium falciparum* 3D7 strain of 44.5 and 43.3 nM.[253,254] The synthetic alcohol derivative **241** was the most active with an IC_{50} of 24.1 nM. This natural product was obtained by semisynthesis via Ugi reaction starting from DINCA (**215**). It is interesting to note that the bridgehead isocyanide did not react, allowing for a regioselective Ugi reaction. This feature was later exploited for the generation of a library of hybrid compounds of antimalarial properties, prepared via Ugi reaction using DINCA as the isocyanide component and different isoniazid or chloroquine analogues as amine or carboxylic acid component (Figure 52).

Hybrids **243** and **244** were active, but unfortunately their activity was not balanced. Indeed compound **243** displayed an antimalarial activity of 11 nM (IC_{50} against *Plasmodium falciparum* 3D7, SI = 1721) and an antitubercular activity against *Mycobacterium tuberculosis* H37Rv strain of 15 μg/mL. As a comparison, isoniazide has a IC_{50} = 0.44 μg/mL. Compound **244** had an antimalarial activity of 3.6 nM (IC_{50} against *Plasmodium falciparum* 3D7, SI = 7154) and an antitubercular activity of 52 μg/mL. However, this data is interesting because it highlights that the presence of a single isocyanide group is enough to impart potent antimalarial activity. Compound **244** was indeed 11 time as potent as DINCA and twice as chloroquine (IC_{50} = 6.6 nM). None of the synthesized compounds appeared to be toxic on Vero cells.[255]

The geranyl linalool **245** was isolated from marine sponges of the genus *Halichondria*. No biological activity has been reported (Figure 53).[182,256]

A series of cinnamic acid derivatives were isolated from the sponge *Botrylloides tireum*. The botryllamide J (**246**) and H (**247**) were claimed to contain for the first time an aromatic isocyanide. (Figure 54).[257] The Authors remain skeptical about this report, as in our hands we have never been able to prepare 2-isocyanophenol derivatives but only the benzo[*d*]oxazole derivatives as products of the isocyanide intramolecular α-addition to the hydroxyl group. However, this class of

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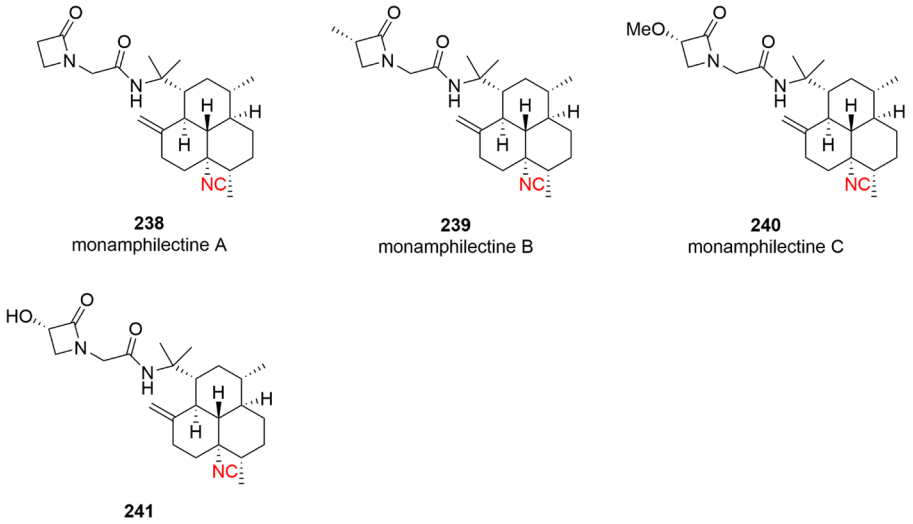

Figure 51. Monamphilectines.

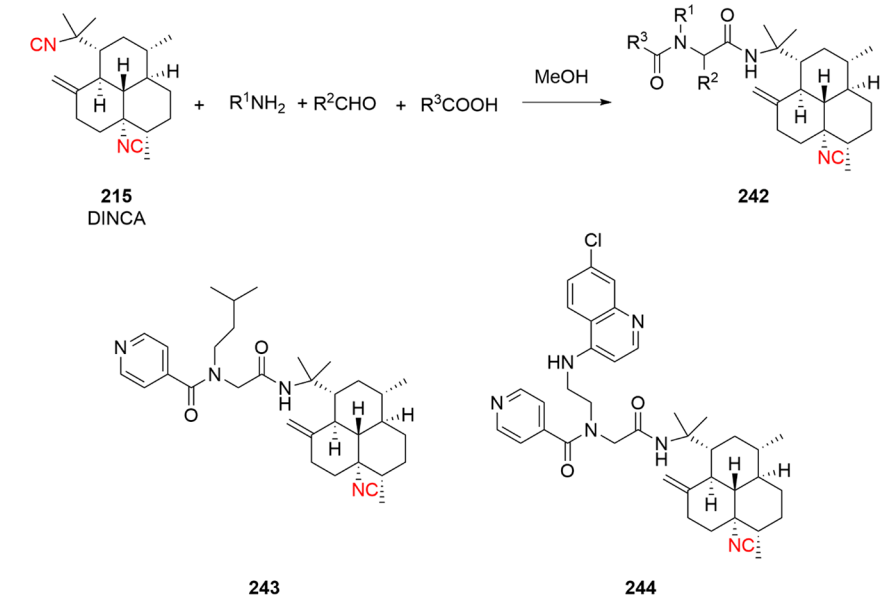

Figure 52. Ugi reaction using DINCA (**215**) as isocyanide input, with structures of hybrids **243** and **244**.

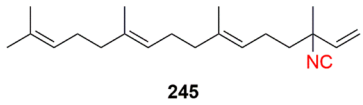

Figure 53. Geranyl-linalool isocyanide.

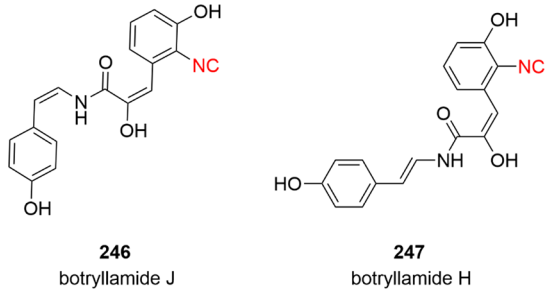

Figure 54. Botryllamides.

compounds is reported to inhibit the multidrug transporter ABCG2. Botryllamide J has an IC_{50} of 26.9 μM.[258]

Finally, isocyanides **248** and **249** (Figure 55) were isolated from the nudibranch *Phyllidia ocellata*. They displayed

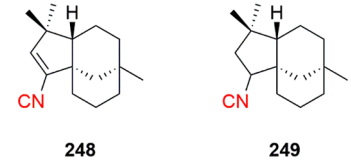

Figure 55. Isocyanides isolated from nudibranch *Phyllidia ocellata*..

antimalarial activity against *Plasmodium falciparum* Dd2 (IC_{50} 0.36 and 0.83 μM respectively) and 3D7 (IC_{50} 0.30 and 0.29 μM respectively), without showing toxicity against mammalian cells (NFF cells).[259]

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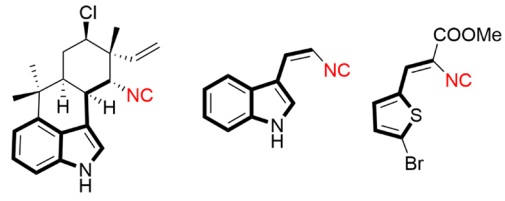

Figure 56. Simplified analogues retain biological activity.

4. BIOLOGICAL ACTIVITY OF SYNTHETIC ISOCYANIDES

As previously described, several natural isocyanides have been discovered. Unfortunately, the biological activity evaluation was possible for only a limited set of them because of the very low isolated amounts. Their structural complexity prevents a scale up of their synthesis, and although some exceptions are known,[260] mass production of these compounds is not feasible. On the other hand, we have already seen that structural simplification is possible and can lead to simplified analogues owing to similar levels of potency. Just as an example, let us consider the case for hapalindole A **87**. This intricated compound has an antibacterial activity of 3.9 μM on *Staphilococcus aureus*. The related simplified indole **11**, which is a natural product, displays an antibacterial activity of 47.6 μM being 12-fold less active. Finally, the totally synthetic compound **82** maintains levels of activity comparable to those of hapalindole A. (Figure 56). This example shows that contributions from medicinal chemists are needed to design and synthesize simplified analogs of natural bioactive isocyanides.

In this secion, we report the synthetic isocyanides which are endowed with biological activity. We decided not to limit the group to synthetic isocyanides from the medicinal chemistry realm but to include examples from the agricultural and antifouling fields, providing a fuller picture around this class of molecules.

4.1. Antibacterial and Antifungal

Due the well-known antibacterial activity of several natural isocyanides, simplified molecules containing an isocyanide as war-headgroup were synthesized and tested as antibacterial compounds. The starting point of this work was the identification of compound **250** during an HTS campaign, which was able to inhibit bacterial growth at a concentration of 32 μM against a panel of *Staphylococcus aureus* methicillin resistant (MRSA) and *Staphylococcus aureus* vancomycin resistant (VRSA) strains. Removal of the isocyano group or its replacement with a cyano group lead to complete loss of activity. The most active compound was isocyanide **251** with a MIC of 2 μM and no cytotoxicity against mammalian cells (e.g., murine macrophage J774) up to a concentration of 64 μM (Figure 57).[261]

However, compound **251** suffered of some drawbacks. Indeed, it was not able to permeate across Caco-2, cells and it was subjected to an extensive metabolism in the presence of human liver microsomes (e.g., 24% remains after 1 h).

In a following paper, other 20 stilbene like mono and di-isocyanides were synthesized and tested. Four compounds (**253**−**256**) emerged from this screening as potent antibacterial compounds, able to inhibit MRSA at concentrations

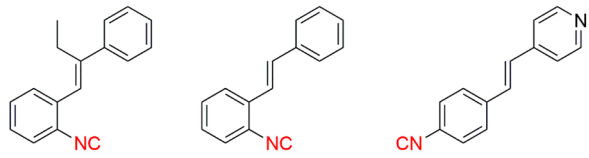

Figure 57. Stilbene isocyanides endowed with antibacterial activity.

between 2 and 8 μM with the bis-isocyanide **256** the most potent (MIC = 2 μM against MRSA USA300). Interestingly, the same compound was active against other pathogenic Gram positive bacteria such as *Staphylococcus epidermidis* and *Streptococcus pneumoniae* while inactive against Gram negative bacterial pathogens (e.g., *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa*). Although in silico pharmacokinetic predictions were reported in the paper, no metabolic studies in vitro have been disclosed rendering difficult a conclusive evaluation (Figure 58).[262]

In a novel study, compound **252**, which was previously synthesized and tested,[261] was shown to maintain a high antibacterial activity against MRSA and VRSA strains with an MIC of 4 and 8 μM, with null toxicity on mammalian keratinocytes. This compound was permeable to Caco-2 cell and demonstrated a good hepatic metabolic stability with a half-life of 11 h. Interestingly, bacterial resistance to this compound was not observed after ten serial passage. Used in a neutropenic thigh infection mouse model at a concentration of 20 mg/kg (two dose) a 75% reduction of infection was recorded, against a 92% reduction obtained with linezolid.[263]

Selected compounds also showed antifungal activity against *Candida albicans* and *Cryptococcus* and *Aspergillus* without being toxic against mammalian cells even at concentration >256 μM. The most potent compound for this set displayed an SI of 512 (Table 22).[264,265]

A simplified SAR for these isocyano stilbene derivatives as antibacterial and antifungal agents is represented in Figure 59.

Among a panel of derivatives of 2-phenylethylamine tested as inhibitors of the growth of attaching bacteria, the isocyanide derivatives **264** and **265** displayed inhibition growth with an IC_{50} of 0.49 and 1.5 $\mu g/cm^2$ (Figure 60).[266]

Synthetic isocyanoalkenes of formula **266** and **267** (Figure 61) were synthesized and evaluated for their fungicidal properties. Some of them showed good antifungal activity, especially against *Tricophyton*, *Candida albicans*, and some plant molds.[267]

4.2. Antimalarial

In 2002, simple isocyanides were prepared and tested as antimalarial compounds against *Plasmodium falciparum* and *Plasmodium yoelii*. Amantadine isocyanide **272** (ED_{50} of 10.7

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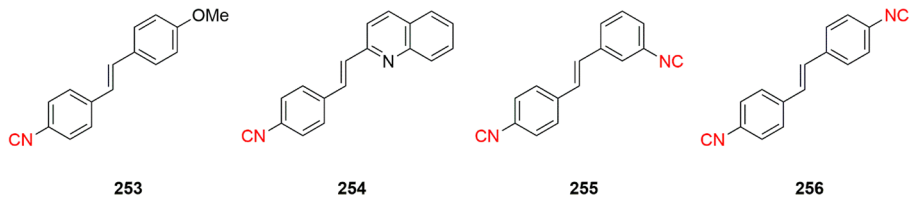

Figure 58. Second generation of stilbene isocyanides endowed with antibacterial activity.

mg/kg) was the most active, with an ability to induce the 100% inhibition of parasitemia after 4 days of treatment using 50 mg/kg on infected Swiss mice (Figure 62). Considering its proved metabolic stability,[13] compound **272** appears as a promising starting point for lead optimization programs (Figure 62).[268]

Simplified analogues (**277**−**279**) of complex isocyanide natural products with antimalarial activity were synthesized along with a nitrile derivative (**280**). The nitrile derivative was inactive, but all the synthesized isocyanides exhibited activity against *Plasmodium falciparum* (Figure 63 and Table 23).[269]

The same stilbene isocyanides (**250**−**263**), previously cited for their antibacterial and antifungal properties, were evaluated as antimalarial agents.

Interestingly, compounds **252**, **256**, and **281** (Figure 64) were the most active against *P. falciparum* Dd2 strain, with EC_{50}'s of 88, 68, and 27 nM, respectively. This data is extremely important, as it highlights how these really simple mono and di-isocyanides have antimalarial activity levels similar to those of the structurally complex 7,20-diisocyanoadociane (**228**; EC_{50} = 13 nM W2) and kalihinol A (**166**; EC_{50} = 1.2 nM FCR-3), while maintaining a very high selectivity index.

The isocyano group is pivotal for the antimalarial activity of these stilbenes. The mechanism of action for these class of compounds is unknown. It is however possible they could share a similar mechanism of action to the one proposed for the antimalarial marine isocyanides, that is the ability to interact with iron protoporphyrin IX. Author hypothesized that bis-isocyanides could interact with two different heme-iron due to the high density of heme in the parasitic environment. This rationale could explain why symmetrical diisocyanides are such potent compounds against *Plasmodium falciparum*.[270]

4.3. Antiviral

Starting from the well-known amantadine antiviral agent and the replacement of the amino group for an isocyanide, a series of amantadine and amantadine-like derivatives were tested for their aniviral proprieties against H5N1 influenza virus. Interestingly, compound **272** not only maintains antiviral activity, but was more potent than amantadine **282** (Figure 65) with a 10-fold improvement (EC_{50} = 0.487 μM vs 3.9 μM of amantadine) in a MTT assay performed on MDCK cells infected with H5N1 virus. As indicated by the authors, the NH_2−NC replacement increases the lipophilicity of the molecule and it could help the molecule crossing the plasmatic membrane. To confirm if these isocyanides maintain the same biological target of amantadine, the M2 protein channel of the virus, compounds were tested on A/M2 channels expressed in *Xenopusoocytes* using the TEVC technique and evaluated at 100 μM. Amantadine inhibits 91%, of the A/M2 channel activity, while the inhibition level for compound **272** was 60%. This result could suggest that the antiviral activity of these isocyanides is due to different mechanism of action other than the blockage of M2 channels.[271]

Isocyanide analogues of thymine nucleoside were synthesized and evaluated as DNA chain terminator. Interestingly, the compound **290** proved to be cytotoxic with a cytotoxic dose (CD_{50}) of 8 and 20 μM against CEM and MRC-5 cells, while the formamide analogues were devoid of activity.[272] The same compound and the uridine analogue **291** were tested as antiviral drugs against the HIV virus, but they proved to be inactive.[273] Compound **290** showed to be cytotoxic against MT-4 cell, with a CD_{50} of 0.88 μM (Figure 66).[274]

The replacement of the primary alcoholic group with an isocyanide was also attempted on thymidine and uracile nucleosides and evaluated against HIV (**292**−**295**). Once more, the compounds were devoid of any activity.[275] 2-Deoxy-2-isocyano-1-β-D-arabinofuranosylcytosine (**296**) was moderately toxic on different cancer cell lines (IC_{50} between 5 and 90 μM; Figure 67).[276]

4.4. Plant Fungicides

α-Isocyanoacetic acid derivatives (**297**) were evaluated as fungicides against several plant fungi. Many of them were protective against *Cladosporium fulvum*, *Phytophthora infestans*, and *Sphaerotheca fulginea*. Compounds **299** was the most potent, being equally active on the fungicid dinocap against *Sphaerotheca fuliginea* and *Cladosporium fulvum*. Another class of compounds, that is, the α-isocyano-β-phenyl propionamides **298**, was evaluated. Compound **300** was twice as active as dinocap (Figure 68).[277,278]

Prochloraz (**301**) is an imidazole containing fungicide inhibiting the sterol 14 α-demethylase. In a case study, it was employed as the lead compound and the replacement of imidazole for isocyanide (**302**) was attempted. The isocyanide analogue resulted less active than **301**, however the authors pointed to the low metabolic stability as a cause of its reduced activity (Figure 69 and Table 24).[279]

As a series of isocyanides of general structure R-A-CN was reported to display fungitoxic activity, and they could therefore be employed in combating phytopathogenic fungi. Because of their good compatibility with higher plants, this set of molecules could be used to spare crops from plant fungus diseases.[280]

4.5. Insecticides and Acaricides

Isocyanophenyl carbamate (**303**) were tested as insecticides and acaricides. It is interesting to note that the introduction of the isocyano group (**304**) on the insecticidal promecarb (**305**) increases the activity against the Southern armyworm of 10-fold (Figure 70).[281]

Quaternary ammonium salts containing two isocyanides (**306**) were reported in a patent as plant insecticides. As a representative example, compound **307** was protective in a 24 h window at a concentration of 0.2% against the *Myzus persicae*, also known as green peach aphid (Figure 71).[282]

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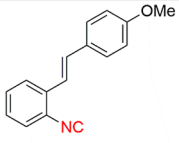
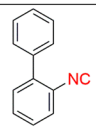
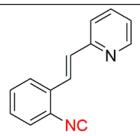
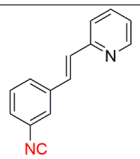
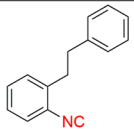
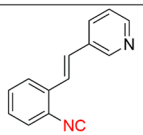
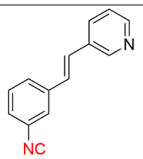
Table 22. MIC (μM) Values for Fluconazole: *Candida albicans* ATCC 29351 < 0.5, *Cryptococcus neoformans* NR-41292 8, and *Aspergillus fumigatus* NR-3501 > 64

Compound	*Candida albicans* ATCC 29351	*Cryptococcus neoformans* NR-41292	*Aspergillus fumigatus* NR-35301
251	1	<0.5	64
252	1	ND	ND
 **257**	1	ND	ND
 **258**	0.5	ND	ND
 **259**	1	<0.5	32
 **260**	1	<0.5	64
 **261**	<0.5	0.5	>64
 **262**	2	1	>64
 **263**	<0.5	<0.5	16

Aliphatic isocyanides with at least 12 carbon atoms are useful as insecticides and acaricides. Specific examples with *n*-dodecyl isocyanide and stearyl isocyanides have been reported.[283]

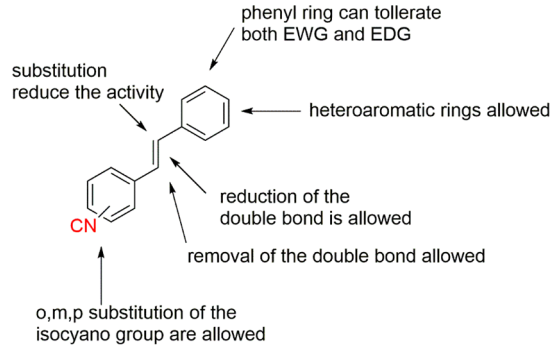


Figure 59. SAR of stilbene isocyanides as antibacterial and antifungal agents.

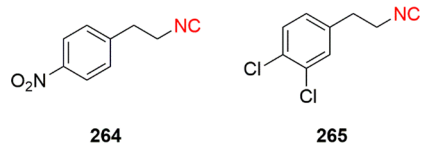


Figure 60. 2-Phenylethylisocyanides as antibacterial agents.

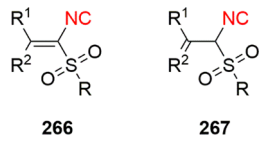


Figure 61. Fungicidal isocyanoalkenes.

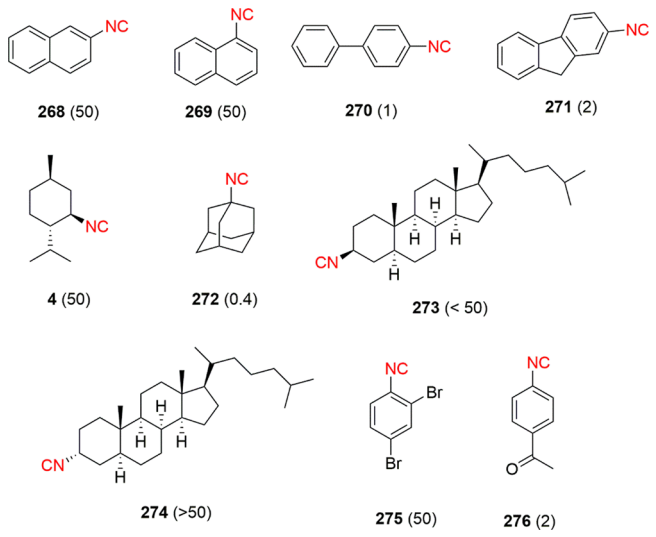


Figure 62. Synthetic isocyanides evaluated as antimalarial compounds. In brackets the MIC (μg/mL) against *Plasmodium falciparum* NF-54.

4.6. Antitumoral

Erbstatin (**293**) is produced by *Streptomyces*. Analogues containing an isocyano group in place of the formaldeyde (**294**−**300**) were reported as tyrosine protein kinase inhibitors.[284] Synthetic formamide analogues displayed higher inhibitory activity against tyrosine kinase (not shown in Table 25) with respect to isocyanides, with the latter maintaining cytotoxic properties on L1210 leukemia cells and antimicrobial activity (Figure 72 and Table 25).

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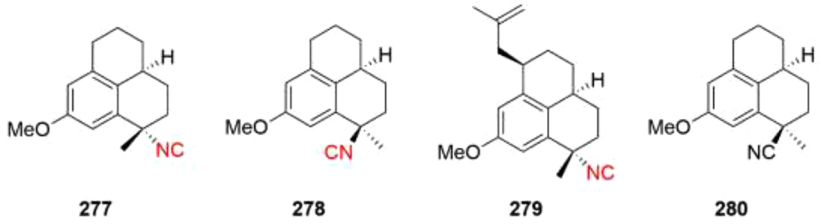

Figure 63. 2,3,3a,4,5,6-Hexahydro-1*H*-phenalene isocyanide derivatives.

Table 23. IC_{50} Values (ng/mL) of Antimalarial Compounds 277−280

compound	*P. falciparum* K1	*P. falciparum* NF54
277	121	102
278	248	169
279	109	100
280	>5000	>5000

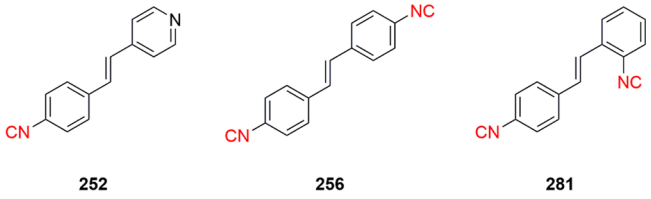

Figure 64. Stilbene isocyanides as antimalarial.

A chemical space of novel *β*-glucuronidase inhibitors was obtained via in silico drug design and claimed in a patent. Inhibition of *β*-glucuronidase represents an antitumoral strategy since this enzyme is overexpressed in several type of cancers. From this set of molecules, the aromatic isocyanide-bearing compound **316** showed and IC_{50} of 15.3 *μ*M and a cytotoxicity against fibroblast 3T3 greater than 30 *μ*M (Figure 73).[285]

4.7. Herbicides

Artificial isocyanides derivatives have also been used in agricultural not only as antifungal and antibacterial agents, but also as herbicides. For example, isocyanoacetamides (**317** and **319**) and ester of isocyanoacetic acid (**318** and **320**)

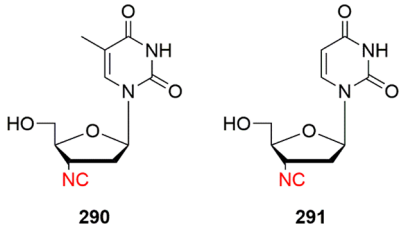

Figure 66. Nucleoside analogues containing an isocyano group.

showed inhibitory action on the growth of broadleaf plants (Figure 74).[286]

The same classes of compounds proved to be active against the germination of rice, cucumber, and radish seeds. The most potent compound was the isocyanoacetic acid anilide **321**.[287] A German patent claimed a role in the regulation of plant growth for the sodium and potassium salts of *α*-isocyanoacetic acids as well.[288,289]

Isocyanoacetamides were reacted with cyclic ketone, and the tertiary hydroxyl group resulting from the addition was dehydrate, furnishing double bond linked exocycloalkyl-*α*-isocyanoacetamides **319**. The synthesized compounds were then evaluated for their germination inhibitory activity against rice, cucumber, and radish seeds as well as for their herbicidal effects on rice, tomato, and weed seedlings. Above all, compound **322** emerged as a selective herbicidal against broad-leaf plants with activities equaling to the commercial herbicides acid 2,4-dichlorophenoxy acetic and 2,4-dichlorophenyl-4′-nitrophenyl ether.[290]

Isocyanide dipeptides **323** were synthesized and evaluated for their inhibitory germination properties at 100 and 10 ppm against rice, cucumber and radish seeds. In this case, the

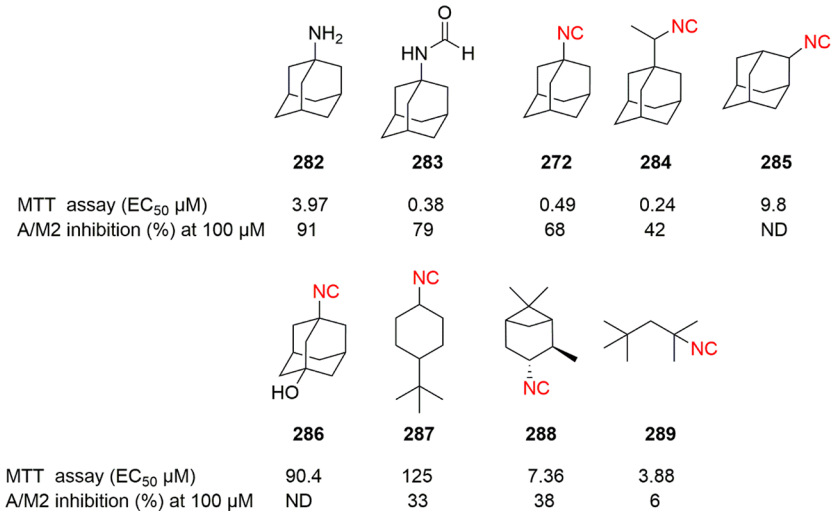

Figure 65. Amantadine derivatives as antiviral agents.

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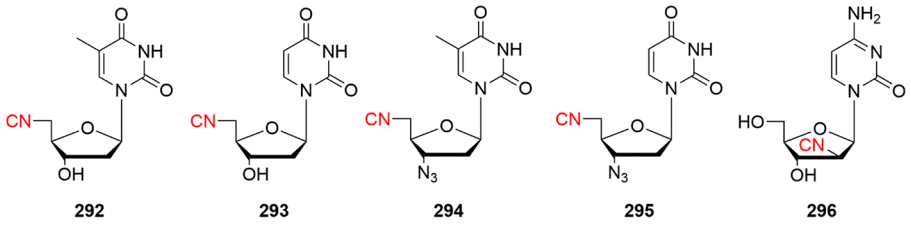

Figure 67. Nucleoside analogues containing an isocyano group.

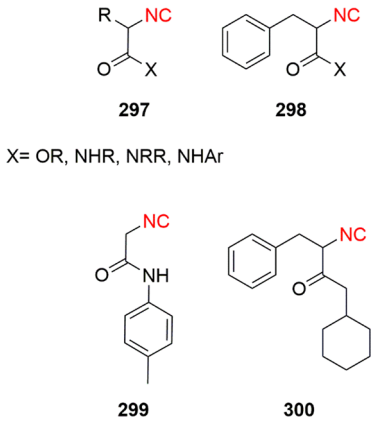

Figure 68. α-Isocyanoacetic acid derivatives as plant fungicide.

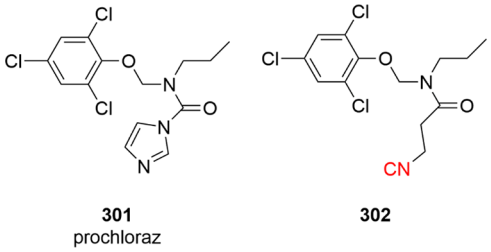

Figure 69. Imidazole replacement with an isocyano group.

Table 24. Biological Activity of Compounds 301 and 302

compound	*B. cinerea* (EC_{50})	IC_{50}[a]	*P. italicum* (EC_{50})	IC_{50}[a]
301	33 nM	2.6 nM	31 nM	8.0 nM
302	17000 nM	100 nM	38 000	920 nM

[a]Inhibitory effect on 4,4-demethyl sterol synthesis.

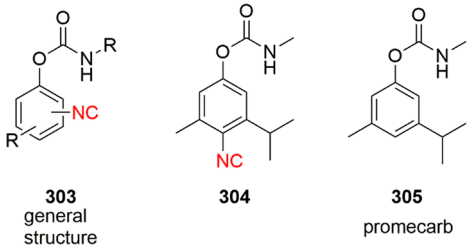

Figure 70. Isocyanophenyl carbamate as insecticides and acaricides.

compounds did not exhibit marked inhibitory effects on germination in comparison with the previous reported α-isocyanoacetic acids (Figure 75).[291]

4.8. Antifouling Agents

The ability of marine organisms, especially mussels, hydroids, and barnacles, to colonize human made marine immersed

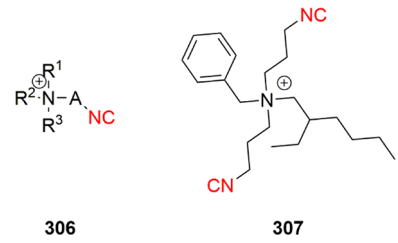

Figure 71. Isocyanide quaternary ammonium salts.

Table 25. Activity of Some Erbstatin Isocyanides

compound	TPK-I IC_{50}, μg/mL	cytotoxicity on L1210 IC_{50}, μg/mL	*S. aureus* MIC, μg/mL	*P. aeruginosa* MIC, μg/mL
309	6.25	1.4	25	50
310	>6.4	0.25	6.25	12.5
311	>10	0.65	3.12	25

structures (e.g., ship hulls, fishing nets, and the cooling systems of power stations) is a well-recognized problem. This characteristic is known as biofouling. Broadly speaking, biofouling is only a part of a greater ecological process known as epibiosis. Epibiosis refers to the colonization of microorganism on living surfaces without entailing symbiosis, parasitism, or commensalism. Notwithstanding, in specific cases this phenomenon can damage the host tissues or impair its ability to get nutrients. Interestingly, many living organisms, especially in the marine world, such as sponges, and soft corals are not subjected to the phenomenon of biofouling. Over the years it was shown that they produce antifouling compounds, preventing biofouling or blocking larval settlement. Their study could be a source identification of environmentally friendly antifouling agents. Indeed, compounds containing tin (bis-(tributyltin) oxide) or copper (copper(I) oxide and copper(II) suphate) are commonly employed as part of protective coatings for ship surfaces to contrast the growth of marine organisms. The use of these metals is currently considered unacceptable from an ecological point of view because of their toxicity against marine organisms and the related introduction in the marine and global food chain. Specific natural compounds containing the isocyano group can suppress the growth of microorganisms and the larval settlement without showing toxic effect on larvae,[223] like kalihinol A from the sponge *Acanthella cavernosa* (EC_{50} = 0.087 μg/mL on *Balanus amphitrite*) and 3-isocyanotheonellin (EC_{50} of 0.13 μg/mL against cypris larvae of the barnacle *Balanus amphitrite*).[292] Both compounds possess antibarnacle activity with a potency comparable to those of copper sulfate. Initially, point modification analogues of 3-isocyanotheonellin (**196**) were synthesized and evaluated (**324**−**326**; Figure 76). All of the synthesized compounds maintained antifouling properties.[293,294]

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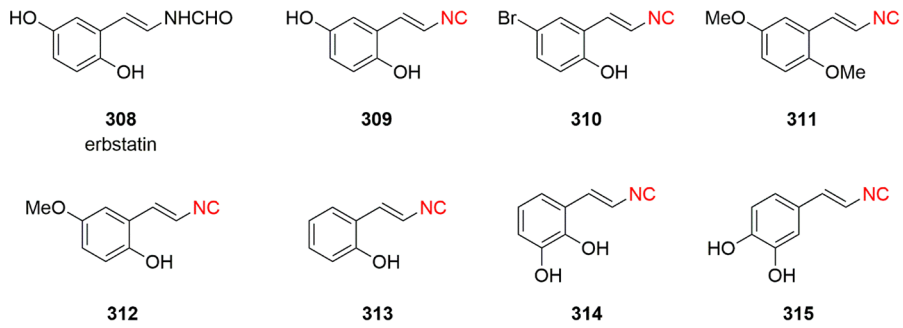

Figure 72. Erbstatin isocyanides.

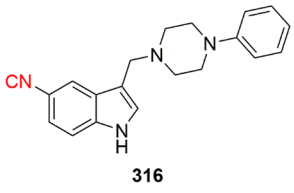

Figure 73. β-Glucuronidase inhibitor containing an isocyanide.

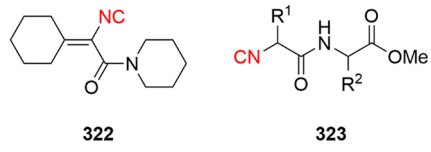

Figure 75. Isocyanoacetamides which inhibit the germination of rice, cucumber, and radish seeds.

Considering the highly chemical complexity of these compounds, structurally simplified synthetic isocyanides were prepared and tested, reveling that simple linear alkyl isocyanide possess similar levels of antifouling activity. The first set of 12 novel linear isocyanides was synthesized and evaluated for its antifouling properties. The larvae settlement was inhibited at concentration between 0.046 and 1.90 μg/mL with a toxicity (LD_{50}) of 21.28 μg/mL, with compound **327** being the best performer (EC_{50} = 0.046 μg/mL; Figure 77).[295]

The dansyl, hence fluorescent, modified compound **328** was employed as a probe in order to observe via fluorescence microscope in which district of *Cypris larvae* the compound was accumulated. Although to less extent than the most potent artificial isocyanide previously described, compound **328** maintained good activity against *Balanus amphitrite* (EC_{50} = 2.80 μg/mL) with no toxicity (LD_{50} > 100 μg/mL). Fluorescence microscope analysis showed high compound concentrations on the oil cell area of cypris larvae, a compartment functioning as food storage (Figure 78).[296]

Interestingly, starting from the widely available and cheap chemical commodity citronellol (**329**), the same Authors prepared 20 artificial isocyanides and evaluated them as antifouling agents against *Balanus amphitrite*. The use of citrollelol allows for the introduction of a tertiary isocyano group. As previously shown, tertiary alkyl isocyano compounds were indeed more active than primary or secondary alkyl isocyano groups Interestingly some of the synthesized isocyanides (**330** and **331**) maintained antifouling properties being only 1.7 fold less active than **327** without showing any sign of toxicity against larvae (Figure 79).[297]

In order to identify the possible biological target of the most promising antifouling isocyanide agent **327**, its activity was evaluated on three marine organisms: *Bugula neritina*, *Balanus amphitrite* (two well-known, taxonomic distant marine fouling organisms), and *Danio rerio*, a zebrafish not involved in the settlement of marine devices. Using the polymer bound compound **332**, it was possible to highlight via SDS-PAGE-LC-MS/MS techniques that the compound binds to three proteins of *B. neritina*. Specifically, two are similar to voltage dependent anion channels (VDAC), which are located on the outer part of mitochondrial membrane and are involved in cell metabolism and cell survival. The third one remains unknown (Figure 80).

The target protein for *B. amphitrite* was a cytochrome P450 and similar to a NADH-ubiquinone oxidoreductase-like protein, a mitochondrial enzyme located into the inner part of the mitochondrial membrane, which is involved in the oxidative phosphorylation, where it catalyzes the electron transfer from NADH to coenzyme Q. Although is unknown which cytochrome P450 isoform interacted with the isocyanide, the authors speculated on similitudes with the CYP15A1 of *Tribolium castaneum*, a beetle species, involved in the insect hormone biosynthesis by catalyzing the transformation of methyl farnesoate into the juvenile hormone III. Finally, the presence of compound **327** in the zebrafish embryo causes the typical signature due to copper deficiency (e.g., pericardial edema, poor blood circulation, pigmentation defects, andf defect on hematopoiesis).[298]

Aromatic isocyanides with a hydrophobic tail at the para position (**333** and **334**) exhibited antifouling properties (Figure 81).[299]

Un-natural amino acids where the α amino group was replaced with the isocyano moiety were prepared and evaluated for their antifouling activity. Interestingly three of them (**335**−**337**; Figure 82) were extremely potent against

Figure 74. Isocyanoacetamides and ester of isocyanoacetic acids as herbicides.

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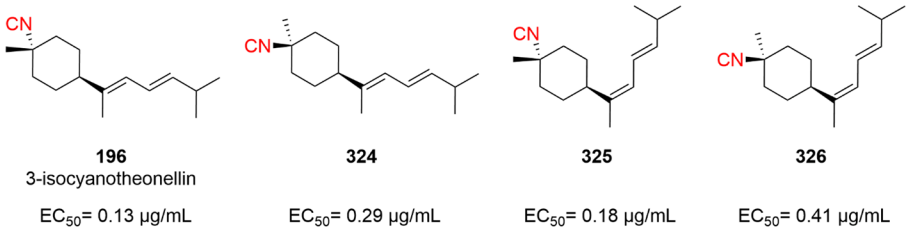

Figure 76. 3-Isocyanotheonellin and analogues.

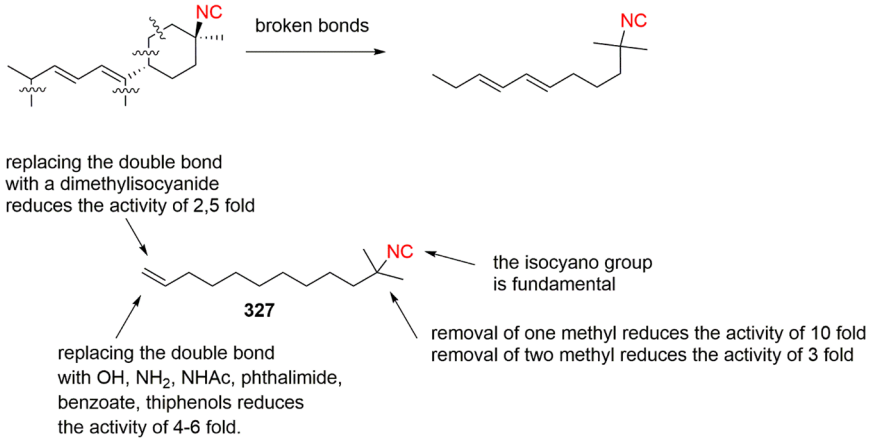

Figure 77. Simplification of 3-isocyanotheonellin and SAR of antifouling agent **327**.

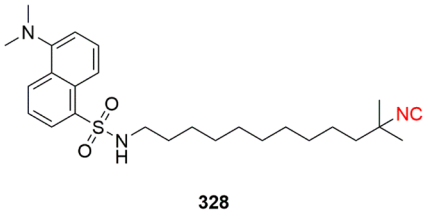

Figure 78. Fluorescent probe.

settlement of cypris larvae *Balanus amphitrite*, without being cytotoxic.[300]

Since the above-mentioned amino acids were obtained in their racemic form, other isocyanide amino acid derivatives containing two identical functional group at the alpha position were synthesized and tested. Even in this case three potent antifouling agents (**338**−**340**) were identified (Figure 83).[301]

Glucosamine isocyanides (**341** and **342**; Figure 84) were proved to exhibit antifouling activity with EC_{50} of 0.23 and 0.25 μg/mL, respectively, against cypris larvae of the barnacle *Balanus amphitrite*, without showing any sign toxicity (LC_{50}) over 10 μg/mL.[302]

5. INTERACTION WITH METALLOPROTEINS

As stated at the beginning of the review, isocyanides are potent metal ligands, and therefore they could be used to perturb

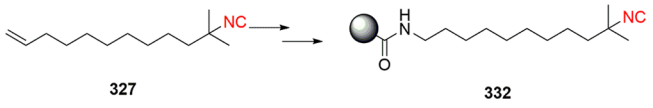

Figure 80. Formation of polymer bound antifouling compound **332**.

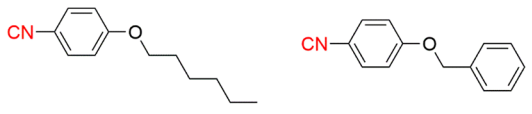

Figure 81. Aromatic isocyanides as antifouling compounds.

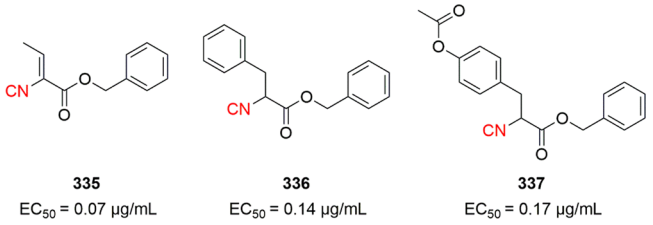

Figure 82. α-Isocyanoacids as antifouling agents.

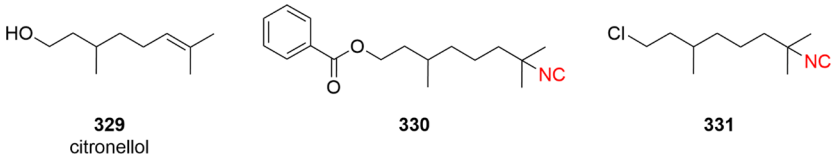

Figure 79. Citronellol and isocyano containing derivatives.

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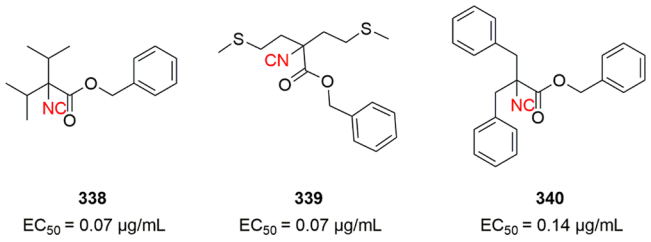

Figure 83. α-Isocyanoacids as antifouling agents.

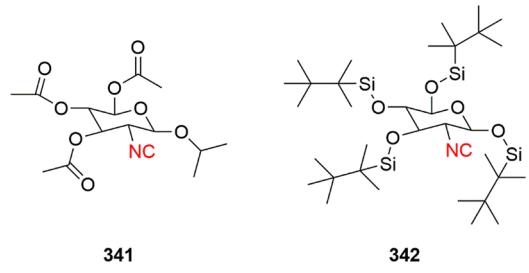

Figure 84. Glucosamine isocyanides as antifouling compounds.

metalloproteins function. Unfortunately, rather than being a feature to capitalize from, this added property has been considered only as a drawback by medicinal chemists, and the studies on the binding of isocyanides with hemoglobin perpetuated the idea of their toxicity. Actually, isocyanides were exploited as tool compounds in many studies on hemoglobin and other metalloproteins, with the goal of defining the topology of their binding site and verifying the effects of ligand binding on the chemical environment around the metal. Those studies were carried out on the purified and isolated proteins, far from a real in vivo context. Just as an example, Professor Malatesta was misled and in his classic paper on metal complexes of isocyanides when he wrote: "They [isocyanides] are extremely toxic, probably because of their ability to combine with hemoglobin".[24] This is apparently not true. Isocyanides are not gaseous compounds like oxygen and carbon monoxide, making the necessary fast cross of membranes and interaction with heme of hemoglobin a far more difficult process to achieve. In this context, it is important to remember that the isocyano group has a large dipole moment (3.85 D) with respect to oxygen (0 D) and carbon monoxide (0.11 D), facilitating the attraction and interaction with the positively charged iron atom of heme. This property finds its empirical correspondence in the better association rate of methyl isocyanide with respect to carbon monoxide in apolar solvents. However, when the heme was dissolved in a detergent based solution mimicking a real-life environment, the effect of the large dipole moment for isocyanide was largely attenuated resulting in similar association constants to heme for the isocyanide ligand and carbon monoxide.[303]

Unfortunately, it is not possible to compare the LD_{50} of carbon monoxide with that of methyl isocyanide due to the lack of data availability for the latter. On the other hand, *tert*-butyl isocyanide toxicity by inhalation was evaluated on rats and mice. Death was caused only at high concentrations with an LC_{50} of 356 and 583 mg/m^3 for male and female rats. Embryotoxic and antispermatogenic effects have been reported.[304] In our view, the in vitro studies for the ability of isocyanides to interact with the metallic prosthetic group of proteins must be seen as the potential demonstration of a putative role for isocyanides as metal binding agents for certain metalloproteins, but not just as warning alert of their potential toxicity.

Linus Pauling was the first to demonstrate the ability of alkyl isocyanides to interact with hemoglobin by competing with oxygen and respecting the same cooperative effect.[305−308] He also demonstrated marked differences of different alkyl isocyanides for their ability to interact with the hemoglobin because of steric hindrance at the heme pocket. Ethyl isocyanide, isopropyl isocyanide and *tert*-butyl isocyanide were evaluated both on free heme and in hemoglobin. In free heme all the isocyanides had the same binding affinity, while large differences in affinity were observed when the compounds were tested on hemoglobin. Indeed, ethyl isocyanide was the best binder compared to isopropyl isocyanide and *tert*-butyl isocyanide. The latter was 200 hundred time less active than ethyl isocyanide (K_d ethyl isocyanide = 0.1 mM, K_d isopropyl isocyanide = 0.3 mM, and K_d *tert*-butyl isocyanide 22.0 mM). The hydrophobic interaction at the heme pocket favored binding, while bulky substituents reduced binding due to steric hindrance. In another paper, 13 alkyl isocyanides were reacted with isolated α and β hemoglobin. Methyl and hexyl isocyanides exhibited the faster association rate, while the propyl isocyanide was the slowest.[309] Aromatic isocyanides are even better ligands for myoglobin and hemoglobin. 2,6-dimethylphenylisocyanide (K_d

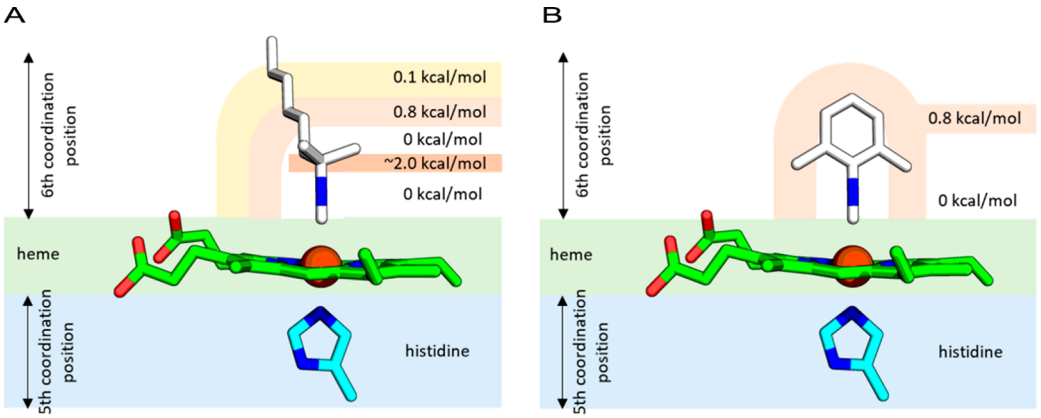

Figure 85. Modern representation of the Reisberg and Olson binding model for aliphatic (A) and aromatic (B) isocyanides in the heme pocket of hemoglobin.

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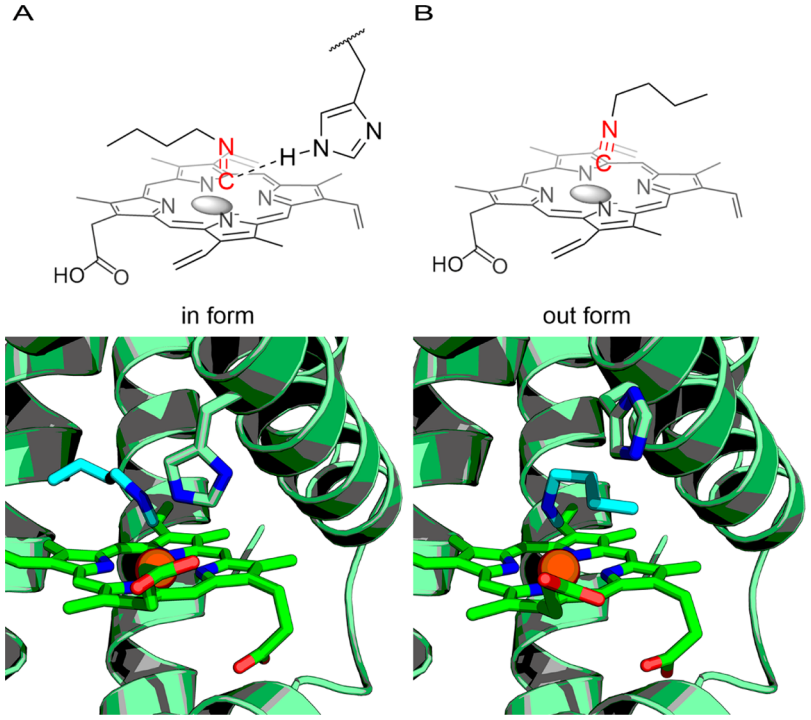

Figure 86. In (A) and out (B) conformations of alkyl isocyanides bound to hemoglobin.[314] X-ray crystal structures 105M and 104M, from Protein Data Bank, were used as in and out example, respectively. Heme structure is depicted as a green stick, butyl isocyanide is shown as cyan sticks, and the Fe ion is represented as an orange sphere.

= 10 nM) and 2,6-diethylphenylisocyanide (K_d = 1 nM) bind to the horse myoglobin with an affinity up to 5000 times greater than ethyl isocyanide. This result ascribes to the important back-donation of the iron ion to the more electronic deficient aromatic isocyanide, which favors the formation of the complex.[310] The affinity of the isocyanides for the iron group also depends on the electron withdrawing properties of the R group. With electron withdrawing groups, such as tosylmethyl, the π acceptor properties of isocyanides are improved, enabling a better and stronger binding between the isocyanide and the metal. The binding to myoglobin is always poor compared to hemoglobin and this can be ascribed to distal steric effects due to the restricted pocket of myoglobin.[311]

We believe it is relevant to repropose the model of Reisberg and Olson regarding the interaction of aliphatic and aromatic isocyanides with the mammalian hemoglobin. The Authors provided a model for the interaction between the iron and the isocyanide in the heme. Four carbon atoms can be accommodated in an unhindered region close to the heme iron. Methyl isocyanide is affected by steric hindrance compared to carbon monoxide. While this contribution could seem marginal at first, the explanation lies in an unfavorable interaction occurring between the isocyanide methyl group and a valinic isopropyl and/or a hystidinic imidazole from the protein chain. Furthermore, the presence of specific distal amino acids can enable or impede the interaction of isocyanides with the heme group. For example, isocyanides bind lesser extent to cytochrome c peroxidases than hemoglobin, and this could be due to the presence of polar amino acids such as Trp, His, and Arg acting as a shield toward the isocyanide, reducing in turn its ability to reach the sixth coordination site (Figure 85).[312]

X-rays showed that isocyanides can interact with heme using two conformations. In the in conformation the alkyl group is directed toward the protein interior with the distal histidine forming a hydrogen bond with the isocyanide. In the out conformation the alkyl group is directed toward the solvent exposed region. (Figure 86).[313]

Alkyl and aromatic isocyanides can interact with the ferrous cytochrome P450 to form a stable complex. In detail, methyl and ethyl isocyanide interact with CYP both in the oxidized and reduced form.[315,316] Ethyl isocyanide complexed with the Fe(III) hemeproteins render a single Soret peak at 434 nm, while Fe(II) hemeprotein complexes return two bands at 430 and 455 nm. This is a common pattern for all the published studies on isocyanides.[315,316] The different binding affinities between isocyanides and the CYP450 oxidation states are shown in Table 26.[317]

Table 26. Dissociation Constant of Isocyanides on Reduced and Oxidized Forms of CYP450

compound	CYP450 (Fe III), μM	CYP450 (Fe II), μM
methyl isocyanide	4700	8.2
ethyl isocyanide	390	8.5
tert-butyl isocyanide	40	6.9
phenyl isocyanide	9.8	4.5

The data in Table 26 highlights the strong binding between isocyanides and the iron heme in its reduced form. However, with the increase of the alkyl chain, more favorable interactions with the Fe (III) state start to arise because of the favorable hydrophobic interactions with the heme frame, resulting in overall lower K_d values.

Isocyanides have a different behavior toward hemocyanins, the proteins involved in the oxygen transport in the

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invertebrates. These metalloproteins contain two copper atoms able to bind to oxygen. Ethyl isocyanide coordinates with the copper atoms preventing and facilitating the expulsion of oxygen.[318]

The metal-coordinating properties of isocyanides can be further exemplified by the ability of cyclohexyl isocyanide to inhibit [Fe]hydrogenase (K_i < 1 nM),[319] the inhibition of methyl isocyanide for nitrogenase (K_i = 158 μM),[320] and of *n*-butyl isocyanide toward carbon monoxide dehydrogenase (K_i = 1.66 mM).[321]

The interaction between isocyanides and indolamine-2,3-dioxygenase has been studied and K_d values were calculated. Ethyl isocyanide, butyl isocyanide and benzyl isocyanide were tested, and their affinities are reported in Table 27.[322]

Table 27. Dissociation Constant of the Ferrous Indolamine 2,3-Dioxygenase with Isocyanides

compound	K_d (μM)
ethyl isocyanide	12.6
butyl isocyanide	0.29
benzyl isocyanide	0.1

The ability of 2,6-dimethylphenyl isocyanide to act as a ligand toward the copper ions complexed in the dopamine-β-monooxygenase was investigated. This enzyme contains a binuclear Cu−Cu center. IR studies showed that, at low concentration of isocyanide, this group can coordinate with only one of the two copper ions in a random manner, and the interaction resulting in two IR bands (2148 and 2129 cm^{-1}). By increasing the concentration of the isocyanide, the previous peaks coalesced into one IR peak (2160 cm^{-1}) still in the isocyanide region, suggesting that both copper ions have been coordinate with two or three isocyanides containing molecules.[323]

The enzyme cystathionine-β-synthase (CBS) is involved in the metabolism of homocysteine. This enzyme contains two prosthetic groups: a) pyridoxal phosphate, b) an iron protoporphyrin IX. When studying the interaction of isocyanide ligand via EPR spectroscopy, if *tert*-butyl isocyanides failed to interact with the ferric heme group of CBS, it would slowly complex with the ferrous heme group of CBS. This complex appeared to be very stable. The rate of interaction is dependent on the nature of the R group, where aromatic isocyanides are more prone to interact with the ferrous heme group of CBS. The relative rate is phenyl isocyanide > benzyl isocyanide = cyclohexyl isocyanide = butyl isocyanide > *tert*-butyl isocyanide.[324]

2,6-Dimethylphenyl isocyanide was shown to interact with the reduced form of peptidylglycine monooxygenase by complexing the methionine-bound copper.[325]

Guanylate cyclase is a heme containing enzyme without the ability to bind oxygen. Butyl isocyanide coordinates the ferrous heme of GC, activating the enzyme by 2−5 fold.[326]

Heme oxygenase is the enzyme responsible for the degradation of heme into biliverdin, iron, and carbon monoxide. Two isoforms are known: the inducible form HO-1 and the constitutive HO-2. HO-1 gained attention from a medicinal chemistry point of view as it is upregulated in oxidative stress and hypoxia. HO-1 has been shown to play an important role in tumor development and progression. Indeed, HO-1 is very often upregulated in tumors and in immune cells such as macrophages and T regulatory cells, highlighting its role in the modulation of tumor microenvironment and cancer cells evasion from the immune response. In fungal pathogens like *Candida albicans* or in bacteria like *Staphylococcus aureus*, HO is primarily used for iron acquisition. It is therefore evident that HO selective inhibitors are potentially anticancer, antibacterial, and antifungal agents. Three isocyanides (isopropyl isocyanide, butyl isocyanide and benzyl isocyanide) were evaluated for their ability to inhibit HO by means of electronic absorption spectroscopy. The results are shown in Table 28.

Heme-oxygenase is characterized by a large hydrophobic cavity close to the point where heme is degraded. It is possible to explain the better inhibitory activity of benzyl isocyanide (K_i = 0.15 μM) by virtue of favorable hydrophobic and π interactions of the phenyl ring, resulting in ligand stabilization. The isocyano group is then able to interact with the heme iron preventing its degradation. The isocyano group can coordinate both with the ferrous and ferric state, however, with a 200 to 300 fold tighter binding for Fe(II). It is interesting to note that benzyl isocyanide does not block the first step of heme degradation, but it coordinates with the verdoheme intermediate, blocking the continuation of heme degradation to bilirubin. In the same work, the inhibitory activity to CYP3A4 was also investigated. All three isocyanides were able to inhibit CYP3A4, with benzyl isocyanide being the better inhibitor with a K_d of 0.40 μM, 15-fold weaker than HO-1.[327]

The reported interaction of TosMic and 2-naphthylisocyanide with the [Fe]-hydrogenase, an enzyme involved in the archaeal methane forming pathway, is an interesting one. This enzyme contains an iron guanylpyridinol cofactor and it is able to bind to molecular hydrogen and favor its scission. TosMic and NIC are both able to inhibit the enzyme by coordinating with iron, by virtue of K_i values of 2 and 10 nM respectively. X-rays analysis of the complex between the enzyme and the isocyanide reveled that the isocyano group was covalently bound to the pyridinol hydroxy oxygen of the cofactor. This C−O covalent bond takes place after the isonitrile coordinates with the iron ion. The formation of this covalent bond could explain the strong inhibitory effect of these two isocyanides. Despite the formation of the covalent bond, the described inhibition was found to be reversible. This fact could be

Table 28. Dissociation Constant and IC_{50} Values in μM of Isocyanides Bound to HO-1 and CYP3A4

comp.	K_d hHO-1 (Fe III)	K_d hHO-2 (Fe III)	K_d HO (Fe III) *C. albicans*	K_d hHO-1 (Fe II)	K_d HO (Fe II) *C. albicans*	K_d CYP3A4 (Fe II)	IC_{50}hHO-1	IC_{50}hHO-2
isopropyl isocyanide	250	170	3800	0.54	4.3	3.7	0.72	1.17
butyl isocyanide	29	33	ND	0.07	0.5	1.3	0.25	0.34
benzyl isocyanide	6	8	ND	0,03	0.03	0.40	0.13	0.10

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ascribed to the severe distortion of the newly formed five-membered ring, which can be released by reconstituting the original isocyanide (Figure 87).[328]

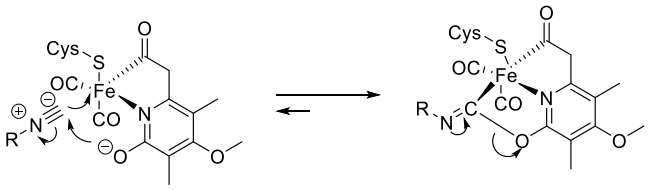

Figure 87. Inhibitory mechanism of isocyanides with the [Fe]-hydrogenase.

Ethyl isocyanide was shown to irreversibly inhibit xanthine oxidase, the enzyme catalyzing the oxidation of xanthine to uric acid. In detail, the isocyano group complexes molybdenum. Interestingly other isocyanides such as *tert*-butylisocyanide and phenyl isocyanide were not able to inhibit this enzyme.[329]

Sterol 26-hydroxylase is a CYP450 (CYP27A1) enzyme located in the mitochondria, able to hydroxylate the position 26, the terminal methyl group of cholesterol side-chain. It has been shown that phenyl isocyanide is able to inhibit 70% of the enzyme at a concentration of 100 μM.[330]

Methyl and ethyl isocyanide bind to the cytochrome *c* of many photosynthetic bacteria. Other isocyanides were tested that were less potent, with cyclohexyl and *n*-hexyl being the worst.[331,332]

6. CONCLUSIONS

In this review article we have summarized the biological activities of both natural and artificial isocyanides to the best of our knowledge. Our hope is for attentive readers to begin considering the isocyano moiety not only as a strategic functional group in multicomponent reactions, polymer, and organometallic chemistry, but also as a pharmacophoric group in medicinal chemistry. Isocyanides have so far received little attention in this field, and this lack of consideration can be ascribed to the following general believes: isocyanides (i) are characterized by an atrocious odor, (ii) have large spectrum of reactivity, (iii) are easily hydrolyzed to formamides, and (iv) finally for the scientist unfamiliar with its chemistry, the isocyanide may be associated with toxicity by way of association with cyanide. Though certain isocyanides might have some of the above-mentioned properties, these should not prevent the application of isocyanide chemistry to other fields of interest, such as medicinal chemistry. Exclusively focusing the attention on such unwanted characteristics may be misleading, as demonstrated by recent findings. Indeed, isocyanides are hydrolytically stable at physiological pH and chemically inert to most abundant nucleophiles in living organism, such as amines, thiols, and alcohols. Our report on the metabolism of a series of isocyanides revealed a strong stability both in plasma and against the oxidative hepatic metabolism for secondary and tertiary ones.[13] This result was corroborated by other metabolic studies on isocyanide containing molecules presented in this review article.[219,263] Finally, a variety of findings have shown minimal toxicity on mammalian cells for several antibacterial and antimalarial isocyanides.

The examples reported in this review article show that the isocyano group can impart marked and specific biological activities and that definite structure activity relationships can be drawn, confirming that the isocyano group is responsible for specific mechanisms of action. In our opinion, a well-trained and sensible medicinal chemist should reevaluate the isocyano functional group as an opportunity, rather than a liability. Furthermore, although most of isocyanides containing molecules were tested as antibacterial, antifungal, and antimalarial agents, it is evident that this functional group can be exploited for other biological targets, thanks to the metal coordinative nature of isocyanides. In many diseases, like cancer and inflammation for example, specific metalloproteins are overexpressed and the exploitation of agents able to elicit their inhibition is a classic strategy. It is therefore clear that novel synthetic isocyanides represent a huge opportunity, since they can be tailored to inhibit specific metal-proteins or against heme containing proteins, both relevant in several diseases or in the survival of pathogens. Isocyanides should be considered on a par with any other functional group used in medicinal chemistry. Drug hunters always felt free to add hydroxamic acids or thiols as warheads in the search of metalloproteins inhibitors notwithstanding their potential toxicity, and in pursuing this strategy, they succeeded in delivering marketed drugs. The imidazole and the 1,2,4-triazole rings are well-known as the best ligands for iron atom in ferriprotoporphyrins, but this has not prevented their use for the identification of selective inhibitors which exploit the differences in the binding pockets of heme containing proteins. We are convinced that the same paradigm will work for isocyanide containing molecules. Furthermore, despite the fact that many synthetic strategies for the preparation of isocyanides have been devised over the years,[333] their preparation can still suffer from drawbacks and there is room for medicinal and organic chemists to improve or invent novel and mild procedures for their synthesis, especially for highly functionalized compounds. For example, tertiary isocyanides are not always easy to prepare, especially from alcohols, as the elimination reaction is a competitive path, and in several cases an excess of trimethylsilyl cyanide must be used.[334−338] The synthesis of molecules containing two or three isocyanide moieties, a prerequisite for metal binding coordination of free iron and copper ions, is always challenging and usually resulting in poor yields. Finally, the identification of a late-stage isocyanide functionalization of complex and densely functionalized molecules is yet to be discovered.

The recent identification of the mechanism of action of xanthocillin shed new light over the antibacterial activity of this fascinating molecule. It would be highly desirable to encourage studies toward the identification of the mechanisms of action for the most potent isocyanide derivatives endowed with antimalarial activity.

Obviously, additional novel isocyanides could be designed and synthesized as agriculturally useful compounds. Moreover, they could be exploited for their metal binding properties in metabolic disorders which raise the concentration of metals, such as copper and iron, to a pathological level.

If the hope is to see an approved drug containing an isocyano group in the XXI century, medicinal chemists will have to appeal to both their knowledge and creativity, together with a bit of courage, and employ them as pharmacophoric groups. To this reason we conclude this manuscript with a sentence of the German philosopher Novalis, which summarizes the scope of this review article on isocyanides in medicinal chemistry. This sentence has been previously cited in the pedagogical book of the Italian writer Gianni Rodari *The*

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Grammar of Fantasy: An Introduction to the Art of Inventing Stories[339] which helps the readers to develop their creative side: "Hypotheses are nets: only he who casts will catch".

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Author Contributions

[§]A.M. and F.B. contributed equally.

Notes

The authors declare no competing financial interest.

Biographies

Alberto Massarotti received his M.Sc. in medical and pharmaceutical biotechnology in 2006 and his Ph.D. in 2009 from the Università del Piemonte Orientale. He is currently an associate professor of medicinal chemistry at the same university. In 2014 he cofounded the start up IXTAL. His main research interests concern the development and application of novel computational methodologies to discover new biologically relevant compounds, especially in the realm of cancer treatment. He is also working on retrosynthetic combinatorial analysis algorithms based on multicomponent reactions and machine learning algorithm to predict metalloprotein targets of isocyanides.

Francesca Brunelli is a final year student of Chemistry and Pharmaceutical Technologies at the Università del Piemonte Orientale, Novara, Italy. She spent her period of thesis in Professor Tron's lab working on the synthesis of novel isocyanides endowed with anticancer properties and in the search of original electrochemical syntheses of isocyanides.

Silvio Aprile graduated in Chemistry and Pharmaceutical Technologies at the Università del Piemonte Orientale (Novara, Italy) and received in 2008 his Ph.D. at the same University. He is currently a postdoctoral research fellow. His research focuses on the optimization of ADME properties of novel biologically active compounds, with an emphasis on drug metabolism. Current fields of interest include the metabolic fate of emerging functional groups in medicinal chemistry (e.g., triazoles and isocyanides).

Mariateresa Giustiniano graduated in 2007 in Chemistry and Pharmaceutical Technologies at the University of Naples-Federico II, and in 2010 she obtained her Ph.D. in medicinal chemistry (Supervisor: Prof. E. Novellino). She was a visiting student in the laboratories of Prof. G. C. Tron (Università del Piemonte Orientale, Novara, Italy) and Prof. J. Zhu (CNRS, Gif-sur-Yvette, Paris). From 2011 to 2016 she held postdoctoral fellowships at University of Naples Federico II, and in 2016 she became an assistant professor (RTDB). In 2018 she got National Academic Qualification as an associate professor in medicinal chemistry. Her research interests focus on the development of new isocyanide-based multicomponent reactions and on the study of isocyanides' reactivity as somophiles in visible-light photocatalytic processes.

Gian Cesare Tron is a Professor of Medicinal Chemistry at the Università del Piemonte Orientale, Novara, Italy. He received his degree in 1994 in Chemistry and Pharmaceutical Technologies and his Ph.D. in Organic Chemistry in 2001 at the Università di Torino (Italy) both under the supervision of Prof. Giovanni Appendino. Over the years, he spent sabbatical leaves in the laboratory of Prof. Jieping Zhu (Institut de Chimie des Substances Naturelles - Gif-surYvette, France), Varinder K. Aggarwal (School of Chemistry − Bristol, U.K.) and Valery V. Fokin (The Scripps Institute − La Jolla, U.S.A.). His personal scientific formation and his views on chemistry resulted from all those different experiences. In 2007 he was awarded the Farmindustria Prize for his achievements in medicinal chemistry. His research interests concern both the discovery of new multicomponent reactions using isocyanides and the medicinal chemistry of anticancer drugs.

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ABBREVIATIONS

3T3-L1, mouse embryonic fibroblast cell line; A2780T, taxol-resistant human ovarian cancer cell lines; A549, carcinomic human alveolar basal epithelial cell line; ABCG2, broad substrate specificity ATP-binding cassette transporter; ACHN, renal adenocarcinoma cell line; B16−F10, murine skin melanoma cells; BE(2)-M17, human neuroblastoma cell line; Caco-2, human colon carcinoma cell line; CBS, cystathionine-β-synthase; CD_{50}, 50% cytotoxic dose; CEM, human T cell lymphoblastic leukemia cell line; CHO, Chinese hamster ovary cells; COMMD1, COMM domain-containing protein 1; COMMD2, COMM domain-containing protein 2; CYP, cytochrome P450; DICA, diisocyanoadociane; DINCA, 8,15-diisocyano-11(20)-amphilectene; DNA, DNA; Du145, human prostate cancer cell line; EC_{50}, half-maximal effective concentration; EDG, electron donating group; EPR, electron paramagnetic resonance; ERK, extracellular signal-regulated kinase; ESI-MS, electrospray ionization mass spectrometry; EWG, electron withdrawing group; FM3A, murine mammary carcinoma cells; Ga, giga annum; GC, guanylate cyclase; GI_{50}, 50% cell growth inhibition; H9c2, rat cardiomyoblasts; HA, hemagglutinin; HCT-116, human colon tumor cell line 116; HeLa, human epithelial carcinoma cell line; HepG2, hepatocellular cancer cell lines; hHO, human heme oxygenase; HIV, human immunodeficiency virus; HMOX, heme oxygenase; HO, heme oxygenase; HS683, human brain glioma cell line; HSPA1B, Heat shock protein family A (Hsp70) member 1B; HSPCA, heat shock protein alpha; HT-29, human colorectal adenocarcinoma cell line; HTS, high throughput screening; HuCCA-1, human cholangiocarcinoma cell line;

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IC_{50}, half-maximal inhibitory concentration; ICSs, isocyanide synthases; IMO, International Maritime organization; ISNs, isonitrile synthases; J774, murine macrophage cell line; KATO III, human gastric cancer cell line; K_d, dissociation constant; K_i, inhibition constant; L1210, mouse lymphocytic leukemia cell line; LC_{50}, median lethal concentration; LC-MS, liquid chromatography−mass spectrometry; LD_{50}, median lethal dose; LLC, Lewis lung carcinoma cell line; LoVo, human colon adenocarcinoma cell line; MCF-7, Michigan Cancer Foundation-7 (breast cancer cells); MDA-MB-231, triple negative breast cancer cell line; MDA-MB-435, human breast carcinoma cell line; MDCK, Madin-Darby canine kidney cells; MEK, mitogen-activated protein kinase kinase; Meth-A, mouse sarcoma cell line; MIC, minimum inhibitory concentration; MRC, Medical research council cell strain 5 (human fetal lung fibroblast cells); MRSA, methicillin-resistant *Staphylococcus aureus*; MT1B, metallothionein 1B; MT1E, metallothionein 1E; MT1F, metallothionein 1F; MT2A, metallothionein 2A; MT-4, human T cell leukemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide; NCI-H187, human non small cell lung carcinoma; NDV, Newcastle disease virus; NFF, neonatal foreskin fibroblasts; NIC, 2-naphthylisocyanide; NMR, nuclear magnetic resonance; P388, mouse leukemia cell line; PC3, prostate adenocarcinoma cell line; PD_{50}, 50% protective dose; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PPM, parts per million; QCY-7701, human hepatoma cell line; RD, human rhabdomyosarcoma; RNA, ribonucleic acid; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; SF-268, human glioma and astrocytoma cell; SI, selectivity index; SK-MEL-28, human malignant melanoma cell line; SNU-398, hepatocellular carcinoma cell line; Sp., Species; TEVC, two-electrode voltage clamp; THP, tetrahydropyran; TosMic, toluenesulfonylmethyl isocyanide; TPK-I, tyrosine protein kinase I; TPO, thrombopoietin; TXB2, thromboxane B2; U373, human glioblastoma astrocytoma cell line; VDAC, voltage dependent anion channels; VRSA, vancomycin-resistant *Staphylococcus aureus*

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