Antimicrobial and Biofilm Inhibiting Diketopiperazines

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Abstract

Diketopiperazines are the smallest cyclic peptides known. 90% of Gram-negative bacteria produce diketopiperazines and they have also been isolated from Gram-positive bacteria, fungi and higher organisms. Biosynthesis of cyclodipeptides can be achieved by dedicated nonribosomal peptide synthetases or by a novel type of synthetases named cyclopeptide synthases. Since the first report in 1924 a large number of bioactive diketopiperazines was discovered spanning activities as antitumour, antiviral, antifungal, antibacterial, antiprion, antihyperglycemic or glycosidase inhibitor agents. As infections are of increasing concern for human health and resistances against existing antibiotics are growing this review focuses on the antimicrobial activities of diketopiperazines. The antibiotic bicyclomycin is a diketopiperazine and structure activity studies revealed the unique nature of this compound which was finally developed for clinical applications. The antimicrobial activities of a number of other diketopiperazines along with structure activity relationships are discussed. Here a special focus is on the activity-toxicity problem of many compounds setting tight limitations to their application as drugs. Not only these classical antimicrobial activities but also proposed action in modulating bacterial communication as a new target to control biofilms will be evaluated. Pathogens organized in biofilms are difficult to eradicate because of the increase of their tolerance for antibiotics for several orders. Diketopiperazines were reported to modulate LuxR-mediated quorum-sensing systems of bacteria, and they are considered to influence cell-cell signaling offering alternative ways of biofilm control by interfering with microbial communication. Concluding the review we will finally discuss the potential of diketopiperazines in the clinic to erase biofilm infections.

Keywords: antimicrobials, antivirals, biofilm inhibitors, cyclic dipeptides, diketopiperazines, fungicides, quorum quenchers

Introduction

Diketopiperazines are the smallest cyclic peptides known, commonly biosynthesised from amino acids by a large variety of organisms, including mammals [1]. The ability of microorganisms to produce diketopiperazines is widespread and published data have shown that about 90% of Gram-negative bacteria produce them [2]. Diketopiperazines have also been isolated from Gram-positive bacteria [3, 4], fungi [5] and higher marine organisms [6]. One of the earliest reports on the formation of diketopiperazines came from Abderhaden and Komm in 1924 [7]. In 1995 Prasad summarized in a review the bioactivity of diketopiperazines [8] and an excellent review on their biological activities and synthesis was presented by Martins and Carvalho [9].

Biosynthesis of cyclodipeptides can be achieved by dedicated nonribosomal peptide synthetases (NRPS). This is for example the case for the phytotoxin thaxtomin A or gliotoxin. An alternative biosynthetic route uses also NRPSs but produces truncated side products during the synthesis of longer peptides. Examples for this route are cyclo(*D*-Phe-*L*-Pro) **1**, which is released prematurely during the synthesis of the decapeptide tyrocidine A [10] and cyclomarazines A and B released during the synthesis of the heptapeptide cyclomarin D [11]. In 2009 Gondry *et al*. discovered a third biosynthetic route involving a novel type of synthetases which they named cyclopeptide synthases. Their common features are conserved residues in 13 positions comprising the two consensus sequences Hx[LVI][LVI]G[LVI]S and Y[LVI]xxExP, half of which are clustered into two regions. These enzymes use aminoacyl-tRNAs as substrates to catalyze the formation of the diketopiperazine bonds and were found in seven bacterial species of the genera *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Mycobacterium* and *Photorhabdus.* Cloning and expression of these genes into *E. coli* led to the formation of cyclo(*L*-Leu-*L*-Leu) **2** with the exception of the enzyme from *Mycobacterium tuberculosis* which produced cyclo(*L*-Tyr-*L*-Tyr) **3**. Almost all of the compounds produced by these cyclodipeptide synthases were combinations of the four amino acids *L*-phenylalanine, *L*-leucine, *L*-tyrosine and *L*-methionine, with the restriction that the diketopiperazines synthesized by the synthase of *Mycobacterium tuberculosis* always contain *L*-tyrosine, and those synthesized by the others contained *L*-leucine [12]. The enzyme from *Bacillus licheniformis*, YvmC-Blic, was recently crystallized and the structure elucidated [13]. The formation of the corresponding dehydro derivatives from cyclo-dipeptides has been demonstrated by cell free extracts of the albonoursin-producing strain of *Streptomyces albulus*. The restriction was that the diketopiperazines had to contain phenylalanine and an amino acid with an aliphatic side chain to get converted [14].

Important biological activities of diketopiperazines are activities as antitumour [15], antiviral [16], antifungal, antibacterial [17], antiprion [18], antihyperglycemic [19, 20] and glycosidase inhibitor [21] agents. Some of them also show affinities for calcium channels and opioid [22], GABAergic [23], serotoninergic 5-HT1A [24], and oxytocin [25, patents: 26, 27] receptors. A number of studies are directed to important biological activities of diketopiperazines related to the inhibition of plasminogen activator inhibitor-1 [28, 29] and alteration of cardiovascular and blood-clotting functions [30, 31]. It has even been reported that some cyclo-dipeptides can block the development of physical dependence in mice [32]. Recently, it was shown that diketopiperazines are able to activate or antagonize LuxR-mediated quorum-sensing systems of bacteria, and they are considered to influence cell-cell signaling [33, 34]. Furthermore, they have been reported as part of antibacterial nucleosides [35].

As there were several reports and reviews on the activity of diketopiperazines this review focuses on their antimicrobial activities and the readers interested in the other biological activities are forwarded to the reviews cited in this work. We will not only discuss antibacterial, antifungal, antiviral and antiprotozoal activities but we will also put special emphasis on their proposed action in modulating bacterial communication. Concluding the review we will finally discuss the potential of diketopiperazines in the clinic to control biofilm infections.

Diketopiperazines as antibiotics

Resistance of bacteria against antibiotics is a growing problem in the treatment of infections. More and more pathogens develop resistances against many of the antibiotics used in the clinic reducing the possibilities for a successful treatment of the patients. One of the worst cases are vancomycin-resistant Enterococci (VRE) which are sometimes untreatable by any current antibiotic or antibiotic combination. Therefore, discovering antibiotics with anti-VRE activity is essential and a number of screens for natural products have been done. From two *Streptomyces* species cyclo(*L*-Leu-*L*-Pro) **4** (Figure 1), also called gancidin W, was identified which could inhibit the growth of VRE-resistant *Enterococcus faecalis* strains with MIC values at 12.5 μg ml^{-1}. Cyclo(*L*-Leu-*L*-Pro) also showed cytostatic activity at 100 μg ml^{-1} against leukemic cell lines [36, 37]. The combination of cyclo(*L*-Leu-*L*-Pro) **4** and cyclo(*L*-Phe-*L*-Pro) **5** gave an even better effect with MIC of 0.25-1 μg ml^{-1} and was also active against *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Candida albicans* and *Cryptococcus neoformans* with MIC values of 0.25-0.5 μg ml^{-1} [38].

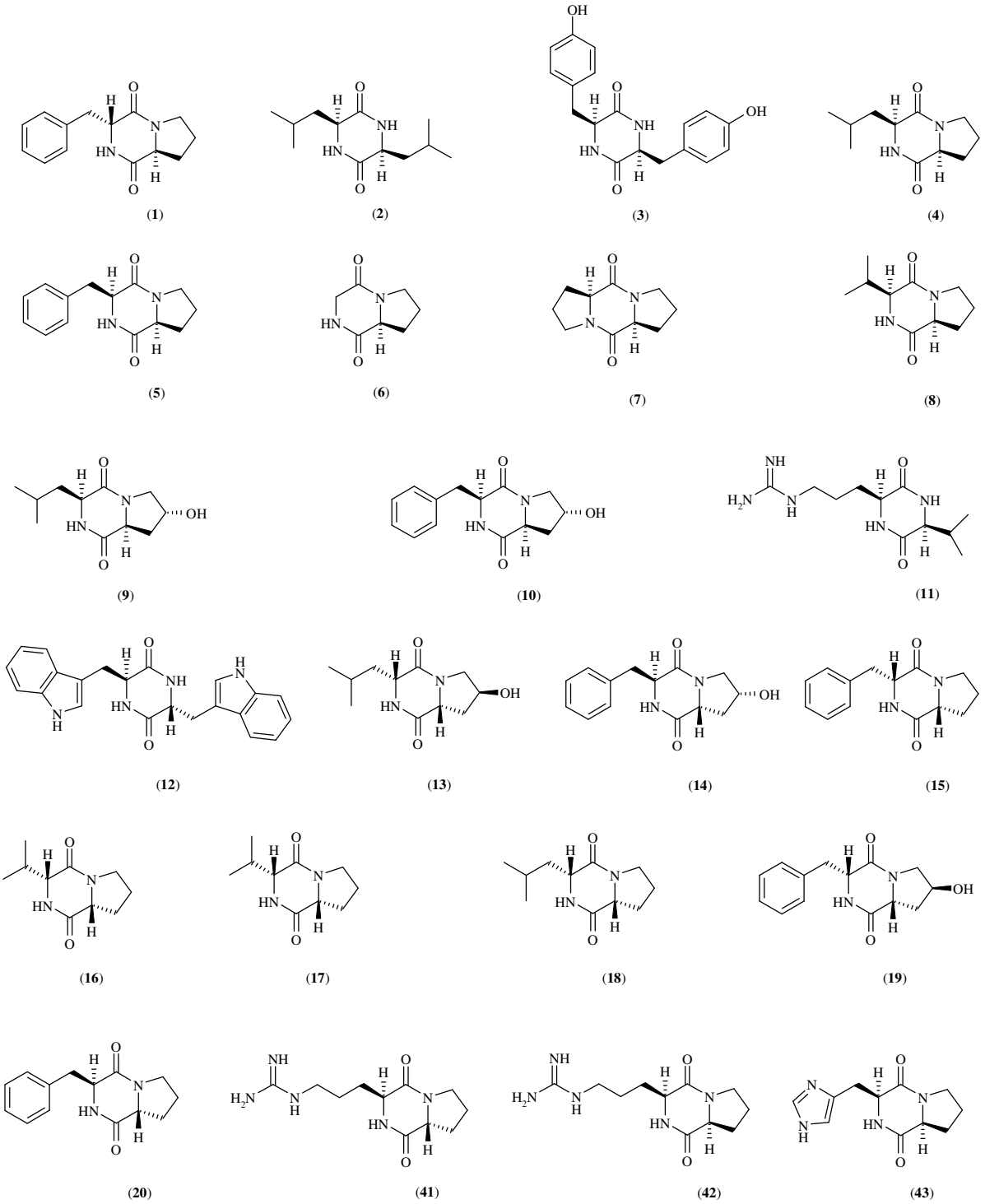


Figure 1: Cyclic dipeptides of reported antimicrobial activities

From the fermentation broth of an *Aspergillus fumigatus* isolate from soil no less than seven diketopiperazines, cyclo(*L*-Leu-*L*-Pro) **4**, cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(Gly-*L*-Pro) **6**, cyclo(*L*-Pro-*L*-Pro) **7**, cyclo(*L*-Pro-*L*-Val) **8**, cyclo(*L*-Leu-*L-trans*-4-OH-Pro) **9**, and cyclo(*L*-Phe-*L-trans*-4-OH-Pro) **10**, were isolated.

All compounds were weakly antibacterial inhibiting the growth of *Staphylococcus aureus* and *Micrococcus luteus* at the concentration of 2.9 mmol l^{-1} [39]. From the blowfly, *Lucilia sericata*, cyclo(*L*-Pro-*L*-Pro) **7** has been isolated and has been demonstrated to have antibacterial activities against *Pseudomonas aeruginosa* and *Micrococcus luteus* [40]. From a *Psychrobacter* species in total sixteen different diketopiperazines have been isolated but only cyclo(*L*-Phe-*L*-Pro) **5** showed a protective effect against *Vibrio vulnificus* and induced cytotoxicity in human intestinal epithelial cells [41]. Cyclo(*L*-Arg-*L*-Leu) **11** was reported from a *Streptomyces* species and found to have antibacterial and antifungal activities [42].

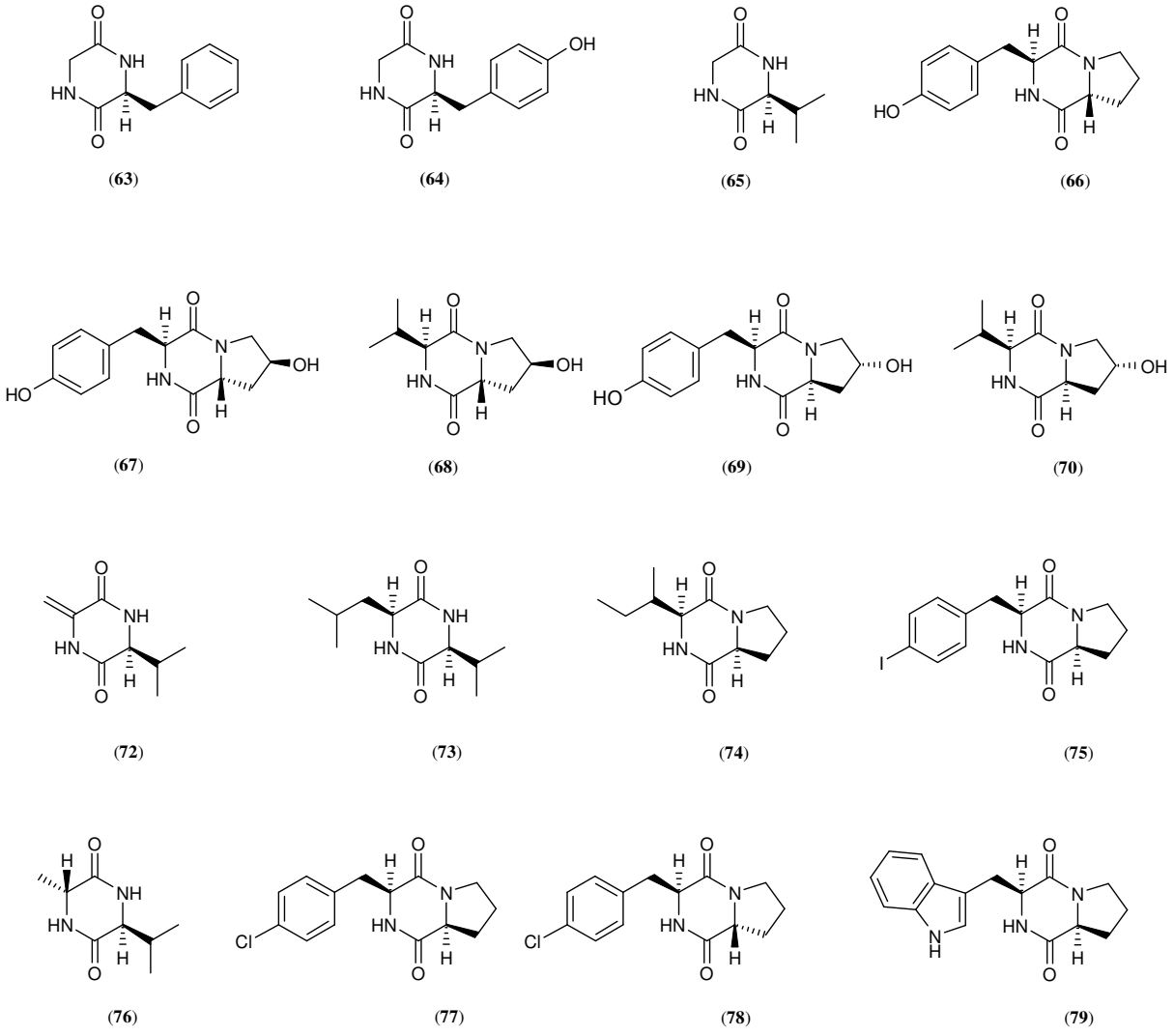


Figure 1 (continued): Cyclic dipeptides of reported antimicrobial activities

Several studies have shown that the absolute configurations of the two amino acids in these piperazines are of importance and a number of *D*-amino acids-containing diketopiperazines have been characterized. Cyclo(*D*-Trp-*L*-Trp) **12** has been isolated from *Penicillium fellutanum* and named fellutanine A [43]. The same

compound has also been isolated from a *Streptomyces* species and shown to be active against clinical multidrug-resistant *Acinetobacter baumannii* strains. Interestingly, the antibacterial activity was only seen for some *A. baumannii* isolates while the growth of others was not hindered [44]. The antimicrobial diketopiperazines cyclo(*L*-Leu-*trans*-4-OH-*L*-Pro) **9**, cyclo(*D*-Leu-*trans*-4-OH-*L*-Pro) **13**, and cyclo(*L*-Phe-*cis*-4-OH-*D*-Pro) **14** were characterized from the actinomycete *Saccharothrix espanaensis* isolated from a marine mollusk [45]. From two unidentified bacteria isolated from the larvae of mollusks five diketopiperazines showing strong antibiotic activity against *Vibrio anguillarum* have been identified. These diketopiperazines, cyclo(*D*-Phe-*D*-Pro) **15**, cyclo(*D*-Leu-*D*-Pro) **16**, cyclo(*D*-Pro-*D*-Val) **17**, cyclo(*D*-Ile-*D*-Pro) **18** and cyclo(*D*-Phe-*trans*-4-OH-*D*-Pro) **19**, all posses the *D*-configuration of the amino acid. The authors performed also structure activity studies and comparisons with other stereoisomers revealing that at least one *D*-amino acid was required for antibacterial activity. Although different combinations of *D*- and *L*-amino acids were also active it seems that higher activity could be achieved by the *D*,*D*-enantiomers. For example cyclo(*D*-Phe-*L*-Pro) **1** had MIC 0.13 and cyclo(*D*-Phe-*D*-Pro) **15** had MIC of 0.03 μg ml^{-1} and the diastereomer cyclo(*L*-Phe-*D*-Pro) **20** showed MIC 0.10 [46].

Beside simple diketopiperazines consisting only of cyclo-dipeptides a number of derivatives are known as bioactive natural compounds. The yeast genus *Rhodotorula* comprises many species forming a red pigment and from *Rhodotorula pilimanae* a derivative of cyclo(*L*-ornithin-*L*-ornithin) was isolated. The metabolite, called rhodotorulic acid **21** (Figure 2), is a dihydroxamic acid and binds strongly iron [47]. Because iron is essential for microbes this compound acts like a growth factor and this may also have an antimicrobial effect in microbial communities. The diketopiperazine antibiotic CJ-17,665 **22** has been isolated from *Aspergillus ochraceus* and was able to inhibit growth of multi-drug resistant *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus faecalis* [48]. This unusual antibiotic contains a bridged diketopiperazine and the rare *N*-oxide moiety. The cyclo(*L*-Ala-*L*-Try) derivative echinuline **23** isolated from *Aspergillus amstelodami* [49] has been found to be active against *Mycobacterium tuberculosis* [50]. From several *Penicillium* species roquefortine C **24** has been isolated. Roqueforine C possesses antibacterial activity against a number of gram-positive bacteria [51] showing MIC of 6.3 μg ml^{-1} against *Bacillus subtilis*. However, the closely related roquefortine E **25** which differs from roquefortine C only by an additional hemiterpene moiety did not show any microbial activity [52]. Cyclomarazines A **26** and B **27** were discovered in the culture broth of *Salinispora arenicola*. They showed moderate antimicrobial activities possessing MIC values of 18 and 13 μg ml^{-1} against methicillin-resistant *Staphylococcus areus* and vancomycin-resistant *Enterococcus faecium*, respectively. Contrary to many other diketopiperazines they had no significant antifungal activity against *Candida albicans* or cytotoxicity against a

human colon carcinoma cell line [53]. An endophytic growing *Gliocladium* sp. isolated from the Amazonian plant *Strychnos* cf. *toxifera* produced the modified diketopiperazine *cyclo*(glycyl-*L*-tyrosyl)-4,4-dimethylallyl ether **28** which exhibited antimicrobial activity against *Micrococcus luteus* with MIC_{50} of 43.4 µM [54].

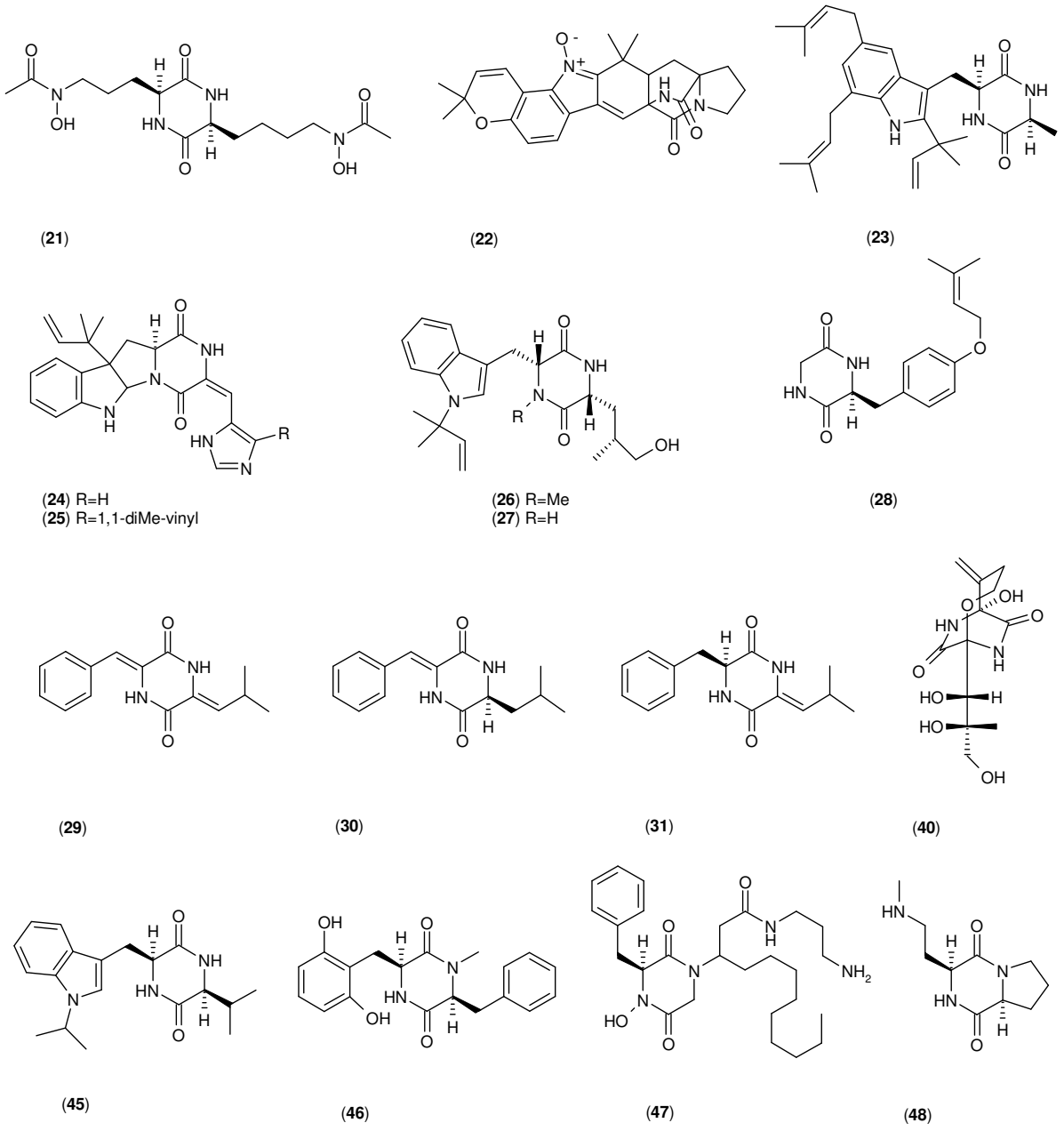


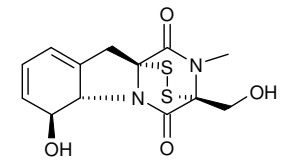
Figure 2: Highly functionalized diketopiperazines with antimicrobial acitivities

Actinomycetes are still a rich source for novel metabolites and from *Actinomyces tumemacerans* the didehydro-diketopiperazine albonoursin **29** has been isolated and characterized. It is antibacterial against a number of *Bacillus* species, but not against *Bacillus subtilis, B. pumilis* or *Klebsiella pneumonia* [55]. Albonursin was also detected in *Streptomyces albulus*, which also produced the biosynthetic intermediates (Z)-3-

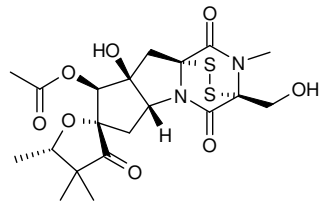
benzylidene-6-isobutyl- **30** and (Z)-3-benzyl-6-isobutylidene-diketopiperazine **31**. Contrary to albonoursin these intermediate had neither antibacterial nor cytotoxic activities, indicating that dehydrogenation at the α,β-positions of both amino acids are required for bioactivity [56].

Many epipolythiodiketopiperazines are known from fungi which are phytotoxins [57]. They comprise well studied compounds like gliotoxin **32** [58], sirodesmin PL **33** or chaetomin **34** [59] (Figure 3). Most of them are also antimicrobial and for sideromin PL antibacterial activities against Gram-positive bacteria [60] and antifungal activities have been described [61]. From *Colletotrichum gloeosporioides* the diketopiperazines bisdethiobis(methylthio)gliotoxin **35** and colletopiperazine **36** were characterized. Only bisdethiobis(methylthio)gliotoxin **35** was active against *Staphylococcus aureus* while the other showed no antimicrobial activities [62]. From a *Microsphaeropsis* fungus the thia-diketopiperazines TAN-1496 A **37**, C **38** and E **39** were identified. The trithia-compound TAN-1496 C was twice as active against most *Bacillus* species tested as its di- and tetrathia-analogues TAN-1496 A and TAN-1496 E but the contrary was found for the activity against *Micrococcus luteus* or *Staphylococcus aureus.* The three compounds were not active against Gram-negative bacteria or fungi but they strongly suppressed the growth of various murine and human tumour cells and induced apoptosis [63]. The antibacterial and antifungal activities of many of these epipolythiodioxopiperazines are interesting but due to their high toxicity in mammals they have not been considered as lead for drug development.

Diketopiperazines comprise also classical antibiotics and one of the best known examples is the unusual natural product bicyclomycin **40** (Figure 2). Bicyclomycin is an antibiotic produced by *Streptomyces sapporonensis* and *S. aizunensis* and has been developed and marketed as bicozamycin by Fujisawa. Its low toxicity (LD_{50} > 4 g kg^{-1} in mice) allows its application as an effective drug against nonspecific diarrhea in humans. It has a unique chemical structure comprising a diketopiperazine moiety [64]. It exhibits activity against a broad spectrum of Gram-negative bacteria with the noteworthy exception of *Proteus* species and *Pseudomonas aeruginosa* and is active against the Gram-positive bacterium *Micrococcus luteus* [65]. The mechanism of action of bicyclomycin is novel because it targets the rho transcription termination factor and bicyclomycin is the only known selective inhibitor of rho. Rho is a member of the RecA-type ATPase class of enzymes that couple oligonucleotide translocation to ATP hydrolysis. The rho protein is integral to the expression of many gene products in *Escherichia coli* and other Gram-negative bacteria, and without rho the cell losses viability due to overproduction of proteins [66]. No clear structure-activity relationship has emerged from the biological activities of bicyclomycin derivates and a Ciba-Geigy group has found that most structural modifications result



(**32**)



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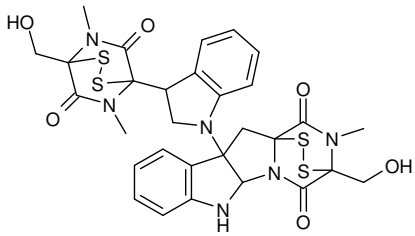

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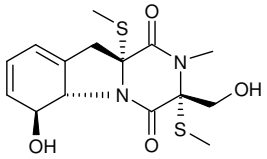
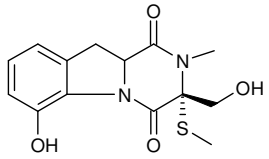
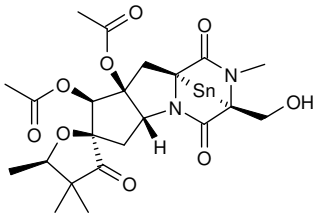


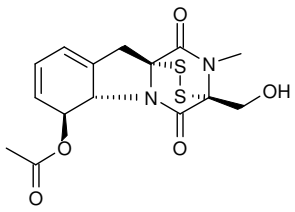
(**35**)



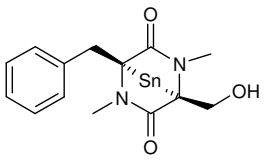
(**36**)



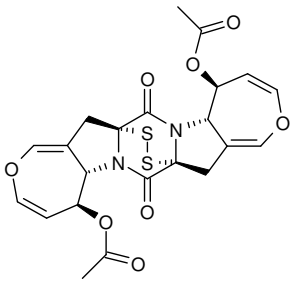
(**37**) n=2
(**38**) n=3
(**39**) n=4



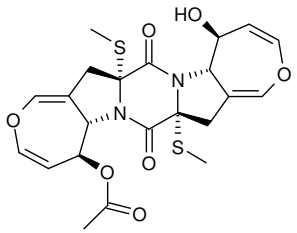
(**49**)



(**50**) n=3
(**51**) n=2
(**52**) n=4



(**53**)



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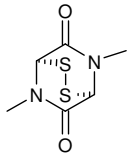

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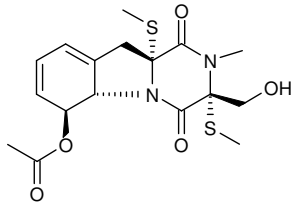
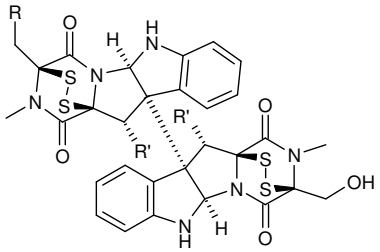
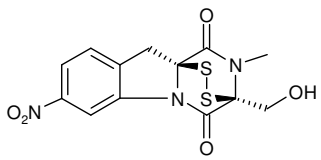


(**56**)



(**61**) R=H, R'=OH
(**62**) R=OH, R'=H



(**71**)

Figure 3: Antimicrobial epipolythioketopiperazines

in reduction or loss of biological activity after preparing a large number of bicyclomycin derivatives [67]. A good overview over the chemistry of bicyclomycin was given by Williams and Durham [68]. It has been shown that the exo-methylene moiety is not a critical element for drug binding to rho [69] but the [4.2.2] bicyclic unit [70] and the triol group [71] are essential. It is interesting to note that cleavage of the isopropyl side chain and benzylation of the amides led to a product of changed specificity. The new derivative showed activity against Gram-positive bacteria like *Micrococcus luteus*, *Bacillus megaterium*, *B. subtilis*, and *Streptomyces cellulosae*, although the activities were weak [72].

Diketopiperazines as antifungal agents

Besides having antibacterial activities many diketopiperazines have been isolated and characterized because of antifungal activities [73]. It has been reported that cyclo(Gly-*L*-Pro) **6**, cyclo(*L*-Arg-*D*-Pro) **41**, cyclo(*L*-Arg-*L*-Pro) **42**, cyclo(*L*-His-*L*-Pro) **43**, cyclo(*L*-Pro-*L*-Tyr) **44** (Figure 1) inhibit the growth of *Saccharomyces cerevisae* by inhibiting family 18 chitinases [74]. An unidentified marine fungus gave cyclo (*L*-*N*-isopropyl-Trp-*L*-Val) **52** (Figure 3) which has antifungal activity against the rice pathogen *Pyricularia oryzae* with MIC 0.36 μM; however, other fungi have not been tested [75]. The two diketopiperazines cyclo(*L*-Phe-*L*-Pro) **5** and cyclo(*L*-Phe-*trans*-4-OH-*L*-Pro) **10** (Figure 1) have been isolated from *Lactobacillus plantarum*. The former possesses weak antifungal activity against *A. fumigatus* and *Penicillium roquefortii* showing MIC of 20 mg ml^{-1} [76]. From the same species cyclo(Val-Val) of unknown stereochemistry has been identified and shown to inhibit *Aspergillus flavus* growth [77]. The derivatised diketopiperazine mactanamide **46** (Figure 2) has been isolated from a marine *Aspergillus* species. It showed fungistatic properties, however, no details have been given in the report [78]. Etzionin **47** has been isolated from an unidentified Red Sea tunicate. It is an unusual diketopiperazine bearing a hydroxamate and a long aliphatic side chain. Etzionin has antifungal properties inhibiting *Candida albicans* with MIC of 3 μg ml^{-1} and showing also inhibition against *Aspergillus nidulans* and the bacterium *Bacillus subtilis* [79] but the compound has also cytotoxic activity. From a *Bacillus* species isolated from a sea urchin five diketopiperazines, bacillusamides A and B, cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(*L*-Pro-*L*-Val) **8**, and cyclo(*L*-Pro-*L*-Tyr) **44** were isolated. Only bacillusamide **48** showed weak antifungal activity against *Aspergillus niger* [80].

The antibacterial activity of many epipolythiodiketopiperazines has been already discussed but many of them possess also antifungal properties. In an interesting *in vivo* model system a number of natural metabolites were tested for antifungal activities. The model comprised the infection of *Caenorhabditis elegans* with the yeast

Candida albicans and allowed the determination of both the antifungal activity and the cytotoxicity of the compounds. In this screen the acetylgliotoxin **49** and epipolypiperazinedione **50** were found to possess the highest activities to protect *C. elegans*. No cytotoxicity was observed for the epipolypiperazinedione **50** [81]. The homologues compounds A26771A (=hyalodendrin [82]) **51**, and A26771C **52** (Figure 3), isolated from *Penicillium turbatum*, were also reported to have antibacterial and antiviral properties but data on the cytotoxicity were not given [83].

Some diketopiperazines have also anti-viral activities

From the fungus *Alternaria raphania* the dihydrooxepine-bearing diketopiperazines acetylaranotin **53**, alternarosin A **54** (Figure 3) bisdethiodi(methylthio)acetylaranotin, and cyclo-(Pro-Tyr) of unknown configuration were characterized [84]. The production of acetylaranotin, here named LL-S88α, was also reported from *Aspergillus terreus* [85] and its absolute configuration was determined by X-ray crystallography [86]. The closely related alternarosin A showed weak antibacterial (MIC 203 µM against *Escherichia coli* and *Bacillus subtilis*) and weak antifungal characteristics (MIC 406 µM against *Candida albicans*) but stronger cytotoxic activities [87]. More interesting is the antiviral activity reported for gliotoxin **32**, acetylaranotin **53** and the synthetic compound *N*,*N*'-dimethylepidithiadiketopiperazine **55** [88]. The antiviral activity of these compounds was reported 4 years earlier by another group which showed also that these compound cannot be used for any clinical applications because of their high toxicity and low water solubility [89]. Gliotoxin **32** and related compounds were reported to display antiviral activities both in their oxidized and reduced forms. Because the antiviral activity disappeared when the reduced state was maintained by the addition of large amounts of reducing agents or after reductive methylation of acetylgliotoxin **49** to **56** the apparent activity of the dithiol was due to cellular oxidation to the dithio-derivative [90].

To develop antiviral drugs one has not to start only from natural products but one can also develop a drug for clinical trials starting from a chemical library. The human immuno-deficiency virus type 1, HIV-1, required the human receptor CCR5 for infection. Therefore, blocking this receptor may offer a novel alternative for controlling HIV infections although other viruses may benefit from such a treatment [91]. Screening a library of spiro-diketopiperazines compound 913 **57** (Figure 4) was identified blocking the binding of macrophage inflammatory protein-1 to CCR5 (IC_{50} 2 nM) inhibiting the replication of some but not all HIV-1 variants [92]. Beside **57** some other compounds from this library showed also promising activities [93] and were developed further. From these studies compound **58** emerged which was five times more active in the binding assay and 50

times more active in the HIV assay than the non-hydroxylated analogue **59**. Remarkable is the tight stereochemical control of the activity. While the S-enantiomer of **59** is three times more active than its mirror compound the contrary is the case for **58** [94]. Intensive analyses of the pharmacokinetic profiles of these and related compounds [95], introduction of a terminal carboxylic acid function together with a structure-activity relationships study [96], led finally to the development of the potent orally available CCR5 antagonist **60** [97, 98].

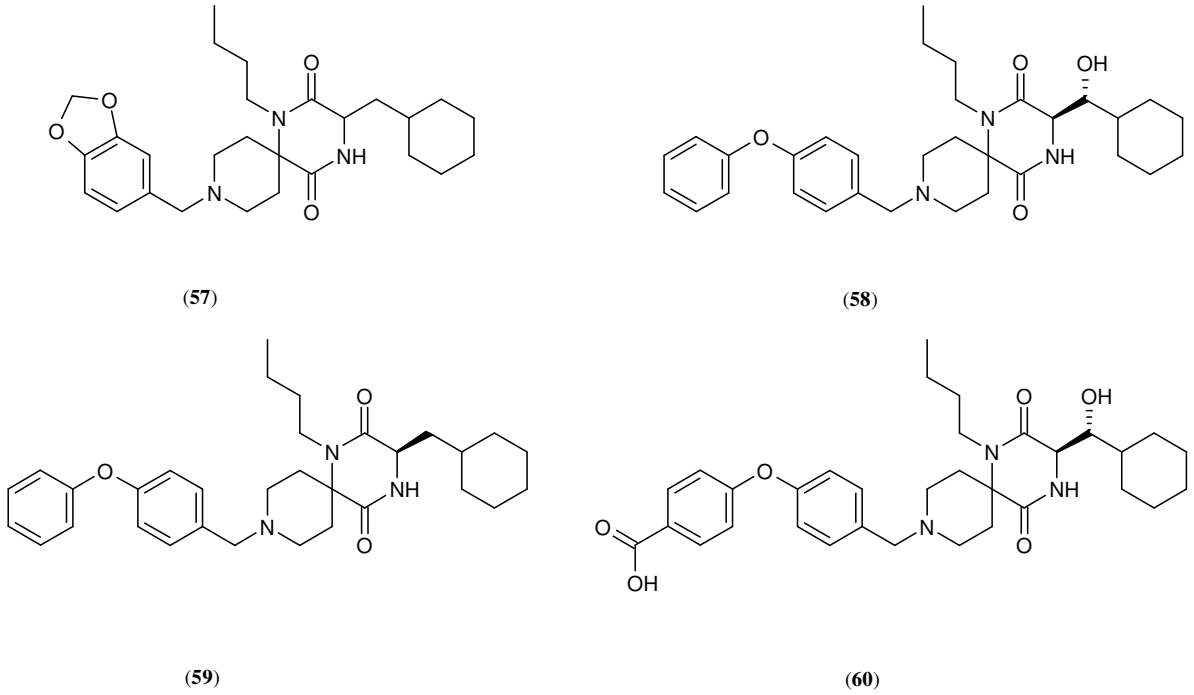


Figure 4: Antiviral diketopiperazines optimized from a library search

There are very few reports on anti-protozoal activities of diketopiperazines. Recently Watts *et al.* screened a number of diketopiperazines for their activities against *Trypanosoma brucei*, the causing agent of African sleeping sickness. For twelve compounds excellent activities have been found and the highest activities were shown by gliotoxin **32**, verticillin B **61** and chaetocin **62** (Figure 3) [99]. From these compounds only gliotoxin showed inhibition of the trypanosomal cathepsin TbCatB, which is essential for the survival of the parasite. The activity of the epipolythiodiketopiperazines against *T. brucei* has, however, to be put into context with their cytotoxicity as mentioned before and any practical use has to be evaluated in this light.

Diketopiperazines as biofilm interfering drugs

Bacteria can adhere to inert or living surfaces, forming structured microbial communities where microorganisms are embedded in a matrix of extracellular polymers (EPS), proteins and DNA, known as biofilms. They represent a protected means of growth that allows survival in a hostile environment and can involve a single microbial species but usually harbor microbial communities [100, 101]. Biofilm formation is associated with several chronic diseases and the results of this colonization are persistent infections that are hard and even impossible to treat, leading to severe health complications and longer hospital stays [102, 103]. Microorganisms can communicate with each other, in a phenomenon known as *quorum sensing* (QS), a mechanism used by many bacteria to perceive and respond to environmental factors. QS is mediated by the production and subsequent recognition of small molecules called autoinducers, e. g. *N*-acyl homoserine lactones (AHL), for Gram-negative and oligopeptides for Gram-positive bacteria [104] .

QS is used to synchronize gene expression and regulate the numerous processes that are involved in community behavior and virulence. The majority of the behaviors controlled by quorum-sensing are much more productive when bacteria express them in synchrony; these behaviors include bioluminescence, EPS production, sporulation, conjugation, and pigment production. However, in pathogenic microorganisms these changes in gene expression allow bacteria also to coordinate the expression of virulence genes and to minimize the impact of the host immune system until a proper number of microorganisms develops in order to establish infection [104,105]. In 2005, Henzer *et al.* compared transcriptomic profiles of *Pseudomonas aeruginosa* growing as biofilm and concluded that it is not possible to deduce if biofilm formation is a QS behavior or an adaptative response to environmental conditions [106]. This finding and those from other researchers were also discussed by Bjarnsholt *et al.* [107]. Nevertheless, the importance of QS in *P. aeruginosa* virulence was shown by Jensen *et al.*, who demonstrated that the bacteria can protect themselves by killing polymorphonuclear leucocytes (PMNs) by the production of rhamnolipid B, a QS controlled behavior [108]. Biomaterial-associated infections are caused mostly by biofilm-forming staphylococci, such as *Staphylococcus epidermidis* and *S. aureus*. These microorganisms, when growing in biofilms are much more resistant to antibiotics than planktonic cells [109]. Usually, patients with this kind of infection are treated with various antibiotics, often without success. Therefore, a replacement of the infected implant is usually required, leading to substantial morbidity and mortality [110, 111].

Essential elements in the role of biofilm formation has been evaluated as targets to new drugs, which includes surface modifications of the medical devices to prevent the bacterial attachment and the use of catheters

with antimicrobial agents. However, the recurrent infections remain frequent, putting emphasis on the need for new compounds effective against biofilms [112].

Interfering with communication

From a *Ruegeria* species associated with a sponge no less than ten different diketopiperazines, cyclo(*L*-Phe-*L*-4-OH-Pro) **10**, cyclo(*L*-Pro-*L*-Tyr) **44**, cyclo(Gly-*L*-Phe) **62**, cyclo(Gly-*L*-Tyr) **64**, cyclo(Gly-*L*-Val) **65**, cyclo(*D*-Pro-*L*-Tyr) **66**, cyclo(*D*-4-OH-Pro-*L*-Tyr) **67**, cyclo(*D*-4-OH-Pro-*L*-Val) **68**, cyclo(*L*-4-OH-Pro-*L*-Tyr) **69** and cyclo(*L*-4-OH-Pro-*L*-Val) **70** (Figure 1) have been isolated [113]. It has been speculated by the authors that they are involved in bacteria - host communication but no experimental proof for this assumption has been shown. *Sphingomonas* species and *Aspergillus fumigatus,* when co-cultured, lead to isolation of the epipolythiodiketopiperazine glionitrin A **71** (Figure 3), harboring a nitro group at the aromatic ring. It possessed significant antibiotic activity against a series of bacteria including methicillin-resistant *S. aureus* and against *A. fumigatus*. Furthermore, *in vitro* cytotoxicity assay revealed that glionitrin A had submicromolar cytotoxic activity against four human cancer cell lines [114]. When challenged by co-culture with another *Bacillus* species the *Bacillus* UA-094 isolate produced among other compounds cyclo-Phe-Pro of unreported stereochemistry [115].

Diketopiperazines, such as cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(*L*-His-*L*-Pro) **43**, and cyclo(*L*-Pro-*L*-Tyr) **44** share structural similarities with endogenous signaling peptides such as the thyrotropin-releasing hormone. Cyclo(*L*-His-*L*-Pro) **43** was already identified in mammalian tissues, and act on the central nervous system, modulating a range of behaviors. Very recently it has been demonstrated that *P. aeruginosa* produces under quorum-sensing control cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(*L*-Pro-*L*-Val) **8**, and cyclo(*L*-Pro-*L*-Tyr) **44**. These three diketopiperazines modulate gene expression in the plant *Arabidopsis thaliana* which influences root architecture [116]. Therefore, bacterial diketopiperazines may have an influence on the eukaryotic host-bacteria, resp. human-pathogen, interactions as well [8].

Diketopiperazines as agonists and antagonists QS autoinducers

A number of reports of interference of diketopiperazines with the QS system have been made, although some of them were conflicting. Cyclo(*L*-Pro-*L*-Tyr) **44** and cyclo(dehydro-Ala-*L*-Val) **72** (Figure 1) have been isolated from the culture supernatants of *P. aeruginosa*. These compounds were also produced by a marine *Micrococcus luteus* strain, *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans.* They were not

found in *Pseudomonas fluorescens* and *P. alcaligenes*, instead, both pseudomonads produced cyclo(*L*-Phe-*L*-Pro) **5**. Four different diketopiperazines, cyclo(*L*-Leu-*L*-Pro) **4**, cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(*L*-Pro-*L*-Tyr) **44**, and cyclo(*L*-Leu-*L*-VaL) **72**, were found in *Pseudomonas putida* WCS358, where cyclo(*L*-Leu-*L*-Pro) **4** and cyclo(*L*-Pro-*L*-Tyr) **44** were able to activate the biosensor strain *Agrobacterium tumefaciens* NT1(pDCI41E33) [117, 118, 119]. Holden *et al.*, showed that the dipeptide cyclo(*L*-Pro-*L*-Val) **8** can activate QS demonstrated by violacein production in the mutant strain of *Chromobacterium violaceum* (CV026) [117]. All three diketopiperazines activate the homoserine quorum sensing biosensor in a concentration-dependent manner, but much higher concentrations were required than for the natural activator *N*-(3-oxohexanoyl)-*L*-homoserine lactone (C6-HSL). Mechanism studies revealed that the three diketopiperazines antagonise the C6-HSL mediated induction of bioluminescence, suggesting that they compete for the same binding site [117]. Cyclo(*L*-Phe-*L*-Pro) **5** produced by *Vibrio vulnificus* was able to activate the quorum sensing on an *E. coli* biosensor strain. This diketopiperazine affects the expression of the *ompU* and *ctx genes,* enhancing the expression of OmpU proteins. OmpU is implicated in several pathogenicity factors, like resistance to antimicrobials and bile acids, organic acid tolerance, biofilm formation, attachment to host cells in symbiotic relationships and adhesion [120]. Diketopiperazines interfering with QS are not limited to bacteria but were also found in archaea in 2011. Five diketopiperazines, cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(*L*-Pro-*L*-Val) **8**, cyclo(*L*-Pro-*L*-Tyr) **44**, cyclo(*D*-Pro-*L*-Tyr) **66**, and cyclo(*L*-Pro-*L*-Ile) **74**, were isolated from the *Haloterrigena hispanica* and it was demonstrated that cyclo(*L*-Pro-*L*-Val) **8** activated the QS system in *Vibrio anguillarum* DM27 shown by the production of the green fluorescent protein Gfp. As already reported in the literature, for the Gfp expression in the *V.anguillarum* biosensor a much higher concentration of diketopiperazine was required than the C6-HSL standard (2.5 mM of diketopiperazine with respect to 50 nM of C6-HSL) [121]. In this context it is noteworthy to remember that some of these diketopiperazines are also known from fungi and plants including the fungi *Rosellinia necatrix,* cyclo(*L*-Phe-*L*-Pro) **5**, and *Alternaria alternata,* cyclo(*L*-Pro-*L*-Tyr) **44**, pointing to an ecological role in species communication.

There are also some reports on QS quenching activities of diketopiperazines. Cyclo(*L*-Pro-*L*-Tyr) **44** and cyclo(*D*-Ala-*L*-Val) **76** reduced colony expansion in one strain of *Serratia liquefaciens*, while cyclo(*L*-Pro-*L*-Tyr) **44** added together with C4-HSL, was able to inhibit swarming behavior of a *S. liquefaciens N*-butanoylhomoserine lactone-dependent swarming motility mutant. Diketopiperazines were demonstrated to modulate quorum-dependent phenotypes by antagonizing the action of acyl homoserine lactones in a LuxR-dependent mode [122]. Diketopiperazines do not only interfere with quorum sensing in Gram-negative bacteria

but have also been reported to do so in Gram-positive ones. For cyclo(*L*-Phe-*L*-Pro) **5** and cyclo(*L*-Pro-*L*-Tyr) **44**, produced by *Lactobacillus reuteri*, it has recently been shown that they strongly inhibit the Ptst promoter of the *agr* system of *S. aureus*. Interestingly, this is the case for all four known agr subgroups of *S. aureus* despite the differences in their autoinducer structures [123].

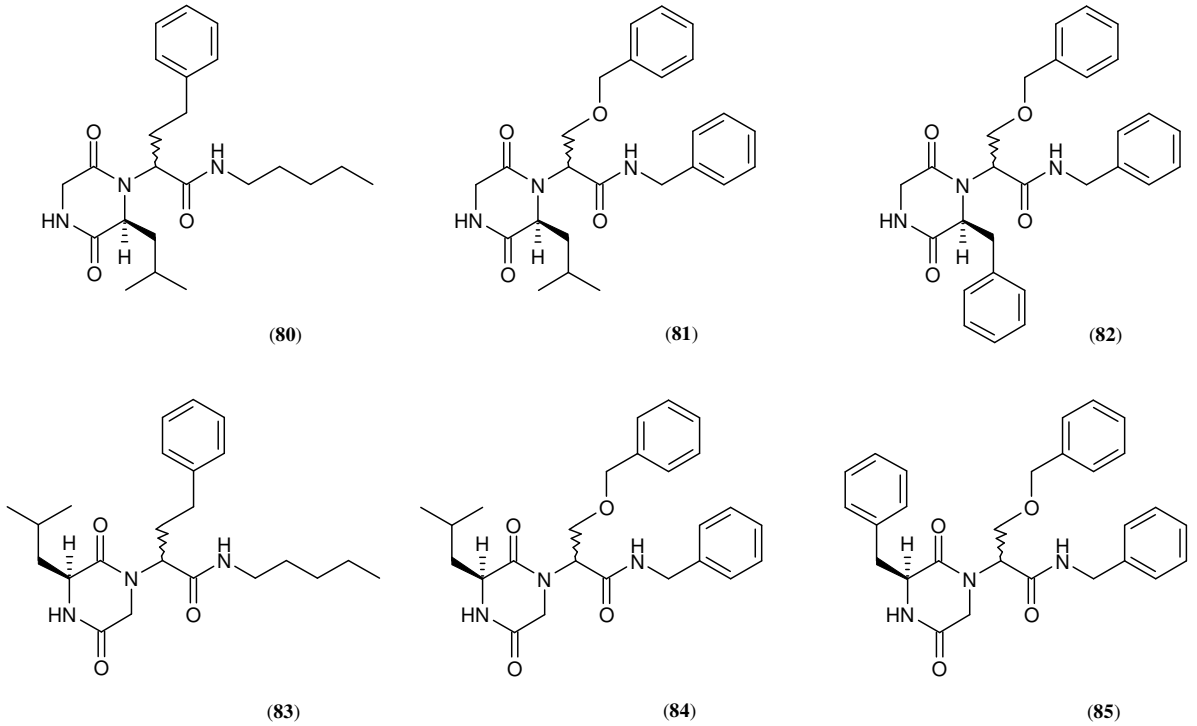


Figure 5: Synthetic diketopiperazines found to inhibit luminescence in *Vibrio fischeri* probably by interfering with quorum sensing

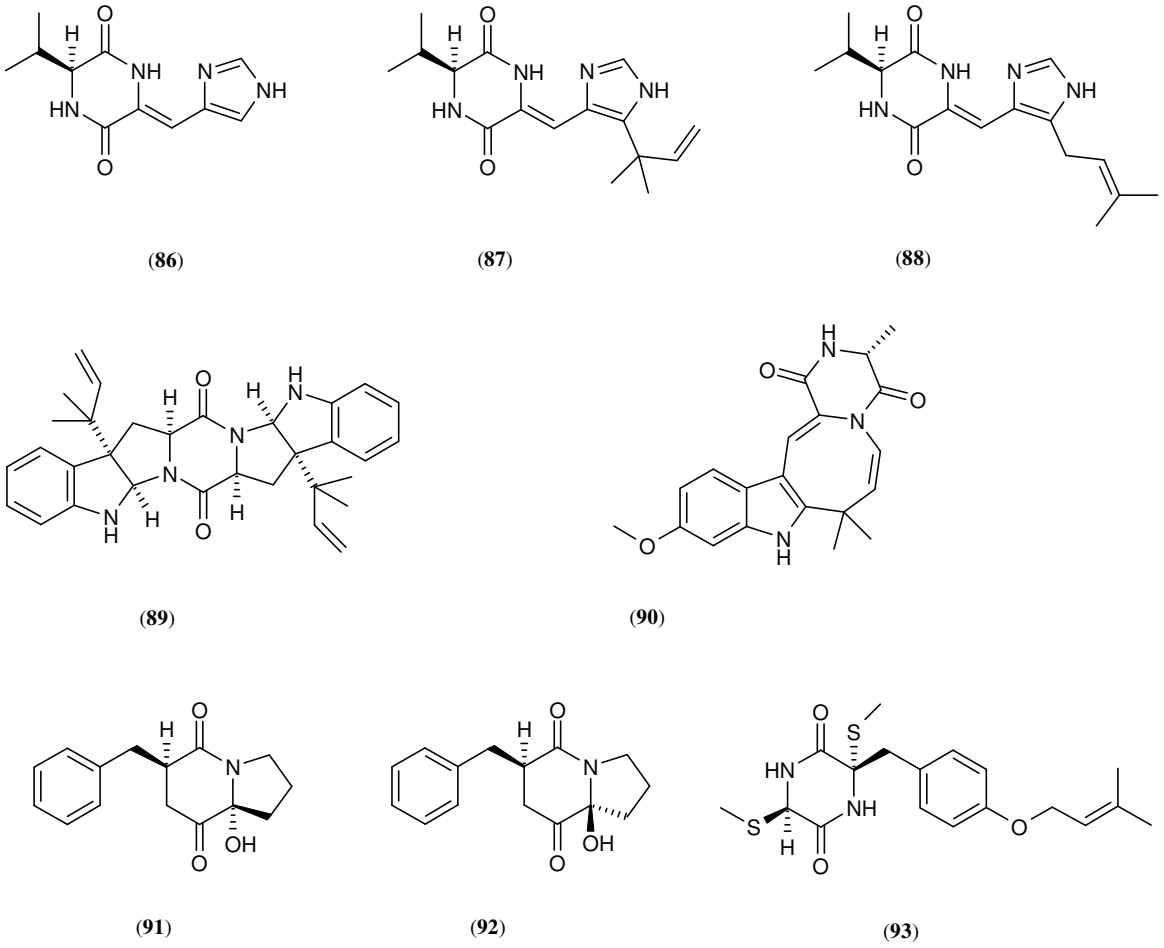
Contradicting these reports that certain diketopiperazines can control QS in bioreporter strains, Campbell *et al*. could not confirm these results. They synthesized several diketopiperazines and tested this library against a *Vibrio fischeri* reporter strain. In this work, the diketopiperazines reported to be active showed neither agonistic nor antagonistic QS activity. However, the authors identified synthetic halogenated cyclo(*L*-Phe-*L*-Pro) derivatives, cyclo(*L*-4-iodo-Phe-*L*-Pro) **75** and cyclo(*L*-4-chloro-Phe-*L*-Pro) **77**, as inhibitor of QS mediated luminescence. Cyclo(*L*-4-chloro-Phe-*D*-Pro) **78** and cyclo(*L*-Pro-*L*-Trp) **79** showed moderate inhibiting activities. Furthermore, they found that these compounds did not compete with AHL for their receptor [124]. This study raises significant doubts that diketopiperazines act as QS signals at least in the test strain used. Nevertheless, at the same time they open the door to systematic structure-activity studies on QS control by diketopiperazines [125]. The authors opened this door further by constructing a diketopiperazine macroarray

consisting of 400 different compounds. They tested these compounds against the luminescence of *Vibrio fischeri*. It turned out that only six of them were inhibitor of the luminescence and none was found acting as agonist. The active compounds **80** - **85** contain either glycine, isoleucine or phenylalanine but similar compounds where these amino acids have been replaced either by alanine or 4-chloro-phenylalanine were not active [126] (Figure 5).

Outlook

Already a large diversity of natural diektopiperazines have been reported but because only still a small fraction of organisms have been explored much more can be expected. The chemical diversity of natural diketopiperazines is high and there are also several recent reports on novel diketopiperazines of unknown functions and activities which widens the diversity of this class of compounds. Recently, the diketopiperazines pre-aurantiamine **86** and aurantiamine **87** have been reported from a marine *Aspergillus aculeatus* isolate [127] (Figure 6). However, no antimicrobial activities have been reported. Aurantiamine is also known from *Penicillium aurantiogriseum* [128]. It is an isomer of viridamine **88** [129] and interferes with the mitochondrial respiratory chain [130]. Also no biological activities are known for the tryptophan-derived isomeric novoamauromine **89** and *ent*-cycloechinulin **90** which were isolated from *Aspergillus novofumigatus* [131]. From the fungus *Chromocleista* species six diketopiperazines of unreported biological activities were identified: the novel epimeric diketopiperazines cyclo(*L*-Phe-2-OH-Pro) **91** and **92** and the known compounds cyclo(*L*-Leu-*L*-Pro) **4**, cyclo(*L*-Phe-*L*-Pro) **5**, cyclo(*L*-Pro-*L*-Val) **8** and cyclo(*L*-Pro-*L*-Tyr) **44** [132]. For Sch 54794 **93**, isolated from a *Nigrospora* species, no antibacterial activity against *Staphylococcus aureus* below 200 µg ml^{-1} could be detected but other bacteria seem to have not been tested [133]. These are some examples of novel diketopiperazines and it can be expected for the future that many more will be identified and hopefully tested for their antimicrobial activities.

There are 20 essential amino acids but the bioactive antimicrobial dipeptides comprise only few of them. There are now a sufficient number of reports of antimicrobial diketopiperazines to make it highly unlikely that this bias is caused only by chance. Obviously nature prefers only a small number of amino acids as building blocks for antimicrobial dipeptides. We see a massive overrepresentation of proline, both as *D*- and as *L*-proline and also as the rather unusual 4-hydroxy-proline. Unfortunately, for the related 2-hydroxy-proline derivatives **91** and **92** no biological activities were reported. While for *cis*-hydroxy-proline only one compound was reported there are four different antimicrobial dipeptides containing *trans*-hydroxy-proline. For a number of amino acids



(**86**) (**87**) (**88**)

(**89**) (**90**)

(**91**) (**92**) (**93**)

Figure 6: Some natural products of unknown biological activity which contain the diketopiperazine skeleton

only the *L*-form was reported but for isoleucine, leucine, phenylalanine, proline, trypthophane and valine both *D*- and *L*-enantiomers gave antimicrobials (Table 1). Antibiotic activities of *DD*- and *DL*-diketopiperazines against *V. anguillarum* were found where cyclo(*D*-Pro-*D*-Val) **17** had a MIC of 0.05µ ml^{-1} and cyclo (*D*-Pro-*L*-Val) of 0.11 µ ml^{-1} [46] . The *LL*-enantiomer was also described in the literature and tested against other bacteria than *V. anguillarum* showing weak activity against *S. aureus* and *M. luteus* [40]. The authors concluded that because of the three diketopiperazine enantiomers (*LL*, *DL* and *DD*) the *DD*-enantiomer showed the highest antibiotic activity *D*-amino acids are more active than their *L*-enantiomers. This fits well to the observation that *D*-amino acids are responsible for biofilm disassembly of *Bacillus subtilis* [134] later also reported for *Staphylococcus aureus* [135]. But comparing other *D*- and *L*-diketopiperazines reported in the literature, no clear connection between the enantiomers and the biological activities can be deduced. A systematic search for all possible combinations of amino acids and their enantiomers could shed more light on the antimicrobial potential of these simple dipeptides. Diketopiperazines from chemical libraries could be relevant to improve our current

knowledge about their biological activities and target interactions, giving rise to new drugs [9]. This could be done by the generation of chemical libraries using approaches already reported in the literature (e. g. for proline derivatives [136]), an approach already applied by Campbell *et al*. in 2009 by using a dipeptide macroarray.

Table 1: Composition of currently known natural antimicrobial diketopiperazines shown in Figure **1**. Note the overrepresentation of *D*- and *L*-proline derivatives.

	L-Leu	*L*-Phe	*L*-Pro	*D*-Pro	*D*-cis-OH-Pro	*L*-tr-OH-Pro	*D*-tr-OH-Pro	*D*-Trp	*L*-Tyr	*L*-Val	*D*-Val
D-Ala										**76**	
L-Arg			**42**	**41**						**11**	
Gly		**63**	**6**						**64**	**65**	
L-His			**43**								
L-Iso			**74**								
D-Iso				**18**							
L-Leu	**2**		**4**			**9**				**73**	
D-Leu				**16**			**13**				
L-Phe			**5**	**20**	**14**	**10**					
D-Phe			**1**	**15**			**19**				
L-Pro			**7**						**44**	**8**	
D-Pro									**66**		**17**
L-Val						**70**	**68**				
L-Trp			**79**					**12**			
L-Tyr						**69**	**67**		**3**		

Concerning bacterial communication, it was reported that cyclo(*L*-Pro-*L*-Val) **8** act as autoinducer and competes with acylhomoserine lactones [121, 117]. However, Campbell *et al*. designed and synthesized several diketopiperazines, both natural and non-natural, and tested them as agonists or antagonists of LuxR, LasR and TraR in a comparison with the literature. The experiments did not confirm this finding and revealed only

activities for the synthetic analogues. The authors hypothesized that this was due to cross talks between different signaling systems [117]. This was confirmed by Park *et al*. showing that cyclo(*L*-Phe-*L*-Pro) **5** affected the expression of the genes *ompU* and *ctxAB* in *Vibrio* species. Currently these conflicting results can be interpreted that diketopiperazines are probably no autoinducers as it was described in the past, but evidences strongly suggest that these compounds can interfere with QS products. However, it should be noted here that all these effects require much higher concentrations than those of the natural autoinducers.

What can be the future for diketopiperazines? For diepipolythio-diketopiperazines many interesting properties have been reported but all reports also caution that these compounds are all too toxic to give useful leads for drug development. One ecological function of a number of natural diketopiperazines seems to be the protection of the producing organism against virus infections. These compounds display indeed antiviral activities but diketopiperazines seems to have no good perspective as antivirals because currently there are better drugs available. The compounds **57 – 60** (Figure 4) may form an exception but the entire approach blocking the human CCR5 receptor for HIV replication but weakening at the same time the human immune system and fostering other viral infections, e. g. hepatitis C, seems to be questionable.

We see an important role for diketopiperazines in controlling biofilm infections. It has already been reported that they are important for interspecies communication and it would be very interesting to determine whether they also influence and shape the communication between pathogens [137] and between the pathogens and the human host. The results on the bacteria-bacteria communications controlled by diketopiperazines are already impressive. They comprise both the suppression of other bacteria and fungi and the induction of the production on bioactive compounds which then interfere with the behavior of pathogens. Currently, these results are still scarce and need systematic exploration and considerable increase of the activities of the antagonists. We are very optimistic that at the end diketopiperazines will be valuable drug to control biofilm infections acting as antibiofilm drugs.

Acknowledgements

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