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Alkaloids: an overview of their antibacterial, antibiotic-enhancing, and antivirulence activities

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Abstract

With reports of pandrug-resistant bacteria causing untreatable infections, the need for new antibacterial therapies is more pressing than ever. Alkaloids are a large and structurally diverse group of compounds, which have served as scaffolds for important antibacterial drugs like metronidazole and the quinolones. In this review we highlight other alkaloids with development potential. Natural, semi-synthetic, and synthetic alkaloids of all classes are considered, looking first at those with direct antibacterial activity and those with antibiotic-enhancing activity. Potent examples include CJ-13,136, a novel actinomycete-derived quinolone alkaloid with MICs of 0.1 ng/mL against *Helicobacter pylori*, and squalamine, a polyamine alkaloid from the dogfish shark which renders Gram-negative pathogens 16 to >32-fold more susceptible to ciprofloxacin. Where available, information on toxicity, structure-activity relationships, mechanisms of action, and in vivo activity is presented. The effects of alkaloids on virulence gene regulatory systems such as quorum sensing, and virulence factors like sortases, adhesins, and secretion systems are also described. The synthetic isoquinoline alkaloid virstatin, for example, inhibits the transcriptional regulator ToxT in *Vibrio cholerae*, preventing expression of cholera toxin and fimbriae, and conferring in vivo protection against intestinal colonization. The review concludes with implications and limitations of the described research, and directions for future research.

Keywords: Alkaloid, Antibacterial, Structure-activity, Mechanism of action, Synergy, Antivirulence

1. Introduction

Antibiotic resistance continues to rise, and with the emergence of pan-resistant untreatable Enterobacteriaceae and *Acinetobacter* spp., the dawn of the much forewarned post-antibiotic era has arguably broken [1]. Improved antibiotic stewardship should help reduce the rate of future losses [2], but antibiotic lifespan is limited even with careful use [3] so this does not negate the need for new anti-infective medications. Biologicals can reduce our dependence on antibiotics and the selective pressure for resistance, but several limitations prevent them replacing antibacterial drugs. For example, safety and efficacy issues preclude vaccine use in severely immunocompromised patients [4], while narrow-spectrum activity precludes monoclonal antibody and phage therapy in infections of unknown aetiology [5,6]. Small molecule drugs, for the time being, remain an essential component of infection treatment and prevention. Two proven strategies within this paradigm are the development of new drugs with direct antibacterial activity (eg. daptomycin) and adjuncts with antibiotic-enhancing activity (eg. tazobactam) [1]. A third as-yet-clinically-unproven strategy is the development of drugs which disrupt bacterial pathogenesis by inhibiting adhesins, autoinducers, and other virulence factors [7].

Historically, natural products have been a rich source of antibacterial drugs. Though the 1980s saw a decline in this type of research in favour of more readily manipulated synthetic compound libraries, this trend is reversing. Synthetic chemical libraries, it is now recognized, tend to be limited in their structural diversity, and a poor source of antibacterial leads [8]. Other factors driving renewed interest in natural products include the discovery of new prokaryotic and eukaryotic species in formerly unexplored environmental niches [9], technological advancements in separation, structure elucidation, dereplication, genome mining, and combinatorial biosynthesis [10,11], and, in the case of medicinal plants and other traditional medicines, concerns that potentially useful medical knowledge and materials are being lost due to urban expansion and species extinction [12]. Efforts to develop synthetic antibacterial drugs have not been abandoned, but are now more focused on derivatization of natural molecules and synthesis of natural product-like compounds using well-known natural product scaffolds [13].

Alkaloids are a large and structurally diverse group of natural products of microbial, plant, and animal origin. Responsible for the beneficial effects of traditional medicines like cinchona bark but also the harmful effects of poisons like ergot, they have a reputation as both Nature's curse and blessing [14]. Alkaloids have inspired the development of several antibacterial drugs, synthesis of quinine serendipitously yielding the quinolones, structural alteration of azomycin yielding metronidazole, and work with the quinoline scaffold yielding bedaquiline. In other drugs, alkaloids are present as scaffold substructures eg. linezolid and trimethoprim. Alkaloids remain the focus of much research, their development as antibacterial drugs pursued within academia, industry, and joint ventures [15-17]. This review seeks to integrate knowledge from the extensive and often widely scattered literature on antibacterial alkaloids. Naturally occurring, semi-synthetic, and synthetic alkaloids are all included provided they are structurally novel with chemotherapeutic or chemoprophylactic potential. Studies with well-characterised pharmacophores already in clinical trials or clinical use have been excluded, as have studies investigating alkaloids as immunomodulators. Structural information on all the described alkaloids is presented in Supplementary Table 1.

2. Occurrence, functions, structure, classification, and nomenclature

Alkaloids are found in bacteria, fungi, plants, and animals, though their distribution within each kingdom is quite limited. They occur in around 300 plant families, specific compounds typically confined to certain families (eg. hyoscyamine in Solanaceae) [18]. Alkaloids can occur in any part of the plant, though specific compounds may be limited to a certain part (eg. quinine in cinchona tree bark) [19]. In terrestrial animals, alkaloids have been reported in insects [20,21], amphibians [22,23], reptiles [24], birds [25], and mammals [26]. Marine animals producing alkaloids include sponges [27], asteroids [28], tunicates [29,30], scleractinia [31], and the dogfish shark [32]. To date, more than 18,000 alkaloids have been discovered [29].

Multiple roles have been attributed to alkaloids in the above organisms, most related to self-preservation, inhibition of competitors, or communication. In microorganisms, for example, alkaloids act

as feeding deterrents [33], allelochemicals, autoinducers, and siderophores [34]. In plants, the inhibitory effects of alkaloids on glycosidase and trehalose metabolism deters herbivores [35], and the ability to quench singlet oxygen confers protection against this toxic photosynthetic byproduct [18]. Alkaloids also act as phytoanticipins and phytoalexins, protecting plants against infection [36]. In the animal kingdom, rove beetles release the surface-active alkaloid stenusine to 'skim' across water away from danger [20], poison dart frogs secrete batrachotoxin as a defence against snake predation [22], and arctiid moths use pyrrolizidine alkaloids as a courtship pheromone [21]. In sponges, alkaloids deter feeding and protect against infection [27], while in scleractinia they act as allelochemicals [31].

Alkaloids are characterized by great structural diversity, the presence of a basic nitrogen atom the only unifying feature [18]. Most alkaloids possess just one nitrogen atom but some have up to five. This nitrogen may occur in the form of a primary amine (RNH_2), a secondary amine (R_2NH), or a tertiary amine (R_3N) [19]. In addition to carbon, hydrogen, and nitrogen, most alkaloids contain oxygen. Alkaloids can occur as monomers, or they may form dimers (also known as bisalkaloids), trimers, or tetramers. Such oligomers are typically homooligomers, but heterooligomers also occur [14].

No single taxonomic principle exists that would allow consistent classification of all alkaloids. Where possible, alkaloids are classified according to chemical structure, biochemical origin, and / or natural origin [14,18]. In terms of chemical structure, there are two broad divisions of alkaloids: the heterocyclic alkaloids (also known as typical alkaloids) which contain nitrogen in the heterocycle, and the nonheterocyclic alkaloids (also known as atypical alkaloids or protoalkaloids) which contain nitrogen in a side chain [18]. Heterocyclic alkaloids are typically classified according to their ring structure (Fig. 1a), though the structural complexity of these sometimes exceeds the number of subdivisions [37]. Nonheterocyclic alkaloids, by comparison, are often divided according to biosynthetic origin. This is possible because their amino acid precursors remain largely intact in the alkaloid structure [37]. Classification according to natural origin is also possible, since specific alkaloids are typically confined to specific sources [18].

Individual alkaloids are assigned names in various ways but almost all names end with the letters '-ine' [19]. Most alkaloids are named after the organism or part of the organism from which they were isolated (eg. atropine from *Atropa belladonna*) [18,19]. When multiple alkaloids are obtained from the same source, a prefix or more complicated suffix is used (eg. quinine, hydroquinine, quinidine) or alternatively a series of letters (eg. epicoccarine A, epicoccarine B) [19,38]. Alkaloids may also be named after the geographic location of their source (eg. tasmanine was isolated from a Tasmanian plant) [14], their pharmacological activity (eg. emetine induces vomiting) or, in some cases, after their discoverer (eg. pelletierine after Prof. Pelletier) [19].

3. Physiochemical, pharmacological, and toxicological properties

Despite their structural diversity, alkaloids share many physical and chemical properties. Because they possess a nitrogen atom with an unshared pair of electrons, alkaloids are basic (hence their name, which literally means alkali-like) [18,19]. The degree of this basicity varies depending on the structure of the molecule and the location of other functional groups. Most alkaloids are solids, but those that lack oxygen (eg. coniine) are liquids. Alkaloids are insoluble or sparingly soluble in water, unless reacted with an acid to form a salt. Alkaloids are soluble in nonpolar solvents like chloroform, but their salts are not [19].

Possessing a proton-accepting nitrogen atom, and one or more proton-donating amine hydrogen atoms, alkaloids readily form hydrogen bonds with proteins, enzymes, and receptors. This, coupled with the frequent presence of proton-accepting and -donating functional groups like phenolic hydroxyl and polycyclic moieties, explains the exceptional bioactivity of the alkaloids [39]. Pharmacological properties include analgesic (eg. codeine), central nervous stimulant (eg. brucine), central nervous depressant (eg. morphine), antihypotensive (eg. ephedrine), antihypertensive (eg. reserpine), antipyretic (eg. quinine) , anticholinergic (eg. atropine), antiemetic (eg. scopolamine) [19], oxytocic and vasoconstrictor (eg. ergometrine) [37], antitumour (eg. vinblastine), and antimalarial (eg. quinine) [18] activities. These activities are exploited in both traditional medicine (eg.quinine-rich cinchona bark in the treatment of

malaria) [37] and modern medicine (eg. vinblastine in the treatment of cancer) [18]. Other alkaloids have been incorporated into human culture as recreational drugs and drugs of abuse (eg. caffeine, nicotine, psilocybin, cocaine) [18,19].

Some alkaloids are highly toxic, and there have been many incidents of human poisonings [18]. In studies of 350 plant-derived pyrrolizidine alkaloids, around half were hepatotoxic, and several carcinogenic [40]. This is due to mammalian liver oxidases transforming the compounds into reactive pyrrole structures which alkylate nucleic acids and proteins [37]. Also, the furoquinolines are phototoxic and photomutagenic, due to the furan double bond reacting covalently with DNA [41]. Other examples include aconitine (cardiotoxic) [42], lycorine (enterotoxic) [43], nicotine (teratogenic) [44], and strychnine (neurotoxic) [45]. Some of the alkaloids used in medicine, at supratherapeutic doses, can be toxic too. Well known historical examples include belladonna (containing atropine) used as a poison in Roman times, and ergot (containing ergometrine) responsible for thousands of deaths in the Middle Ages [19,37].

4. Direct antibacterial activity

4.1 Naturally occurring alkaloids

Studies describing naturally occurring antibacterial alkaloids date back to the 1940s, but much of this early work stopped short of determining MICs. Subsequent research has been more thorough, and several potently antibacterial alkaloid monomers (MICs $\leq$10 μg/mL) have been identified in the aaptamine [46], indole [47-52], indolizidine [53], isoquinoline [54-59], piperazine [60], quinoline [61,62], quinolone [63], agelasine [64,65], and polyamine [32] classes. Alkaloid dimers with similar levels of activity have been found in the aaptamine-indole [66], bisindole [67,68], indole-quinoline [69,70], pyridoacridine [71,72], bispyrrole [73-75], and pyrrole-imidazole [76] classes. A list of compounds with the lowest recorded MICs is presented in Table 1. Toxicity data for these agents is limited but in vitro, chelerythrine [59], hapalindole I [52] and prosopilosidine [53] are antibacterial at concentrations that are nonhaemolytic or nontoxic to Vero cells, and in vivo, squalamine is effective against *Pseudomonas*

aeruginosa pneumonia at 0.15 mg/kg body weight (BW), a dose 65-fold lower than known safe levels [15]. Studies with sanguinarine suggest a daily oral dose of 5 mg/kg BW is safe in animals [77] and, used topically, this agent has proven clinical safety and efficacy in orthodontic patients [78]. The alkyl methyl quinolone alkaloids 1-methyl-2[(Z)-7-tridecenyl]-4-(1H)-quinolone and 1-methyl-2[(Z)-8-tridecenyl]-4-(1H)-quinolone are also worth mentioning here. In combination, these compounds have MICs of 0.02 to 0.05 µg/mL against *Helicobacter pylori*, and proven in vivo efficacy against *H. pylori* infection [79].

4.2 Semisynthetic and synthetic alkaloids

Efforts to improve alkaloid activity through synthetic modifications have been successful when using indole, isoquinoline, pyrrole, and thiazole alkaloids as scaffolds. In the indole class, a β-carboline dimer (NCD9; Supplementary Table 1) and simple indole dimer (5,6,6'-tribromo-1*H*,1'*H*-2,2'-biindole) have been synthesised with respective MICs of 0.1-4.0 µg/mL [83] and 0.5 µg/mL [84] against Gram-positive pathogens. Also, a manzamine A derivative has been produced (8-*n*-hexamidomanzamine A) with an MIC of 0.31 µg/mL against *Mycobacterium intracellulare* [85]. Toxicity tests with CHO-K1 [83], HEK 293 [84], and Vero cells [85] suggest these indoles have selective activity.

In the isoquinoline class, a biphenyl-substituted sanguinarine derivative (compound 9) has been produced with MICs of 0.5 µg/mL against *Staphylococcus aureus* (incl. MRSA) [17]. Also, berberine has been conjugated with a multidrug resistance pump inhibitor to create a compound (SS14) with MICs of 1.8 to 3.7 µg/mL against a range of Gram-positive pathogens [86]. Toxicity of the sanguinarine derivative has not been tested [17], but the berberine derivative is nontoxic at >100 µg/mL and shows efficacy in an in vivo model of enterococcal infection [86].

In the pyrrole class, hybrids of 4,5-dibromopyrrole and 1,3,4-oxadiazole have been produced with MICs of 1.6 µg/mL against *S. aureus* and *Escherichia coli*, and MICs of 1.5-1.6 µg/mL against *Mycobacterium tuberculosis* [87]. Also, a hybrid of 1-methyl-4,5-dibromopyrrole and aroyl hydrazone has been synthesised (compound 4m) with MICs of 0.2 to 0.8 µg/mL against *S. aureus* (incl. MRSA) [88]. Toxicity data for these compounds is not yet available.

Lastly in the thiazole class, a 1,3,4-thiadiazole derivative (compound 6f) has been synthesized with an MIC of 1.56 μg/mL against *M. tuberculosis* [89], and a rhodanine derivative (CCR-11) has been synthesized with an MIC of 1.07 μg/mL against *Bacillus subtilis* [90]. Compound 6f has not been assessed for toxicity, but studies with HeLa cells suggest CCR-11 activity is selective.

4.3 Structure-activity relationships

Structure-activity relationships (SARs) have been investigated for various subclasses of the indole (Fig. 1b) and isoquinoline (Fig. 1c) alkaloids. For β-carboline indoles, dimerization improves antibacterial activity [83], possibly because the larger molecule is less susceptible to bacterial efflux. Amidation of β-carbolines at C-8 reduces toxicity, and if a hexamido group is used, the reduction in toxicity is accompanied by a >20-fold increase in antibacterial activity [85]. Activity also increases with increasing numbers of bromine atoms [51]. For carbazole indoles, hydroxylation at C-1 and isoprenylation at C-4 both improve activity, while methoxylation at C-1 reduces activity [49].

For simple isoquinoline alkaloids, the addition of alkyl substituents at C-1 improves antibacterial activity, but these improvements appear not to be selective [54]. For benzophenanthridine isoquinolines, a methylenedioxy functional group at C-2 and C-3 is important for activity [17,59]. Addition of a phenyl or biphenyl substituent at C-1 or C-12 can improve activity, and further improvements can be achieved by adding a methoxy group at C-7 and C-8 [17]. For protoberberine isoquinolines, a methylenedioxy functional group at C-2 and C-3 improves activity [91], as does a phenoxyalkyl group at C-9 [92].

4.4 Identification of activity as bacteriostatic or bactericidal

Antibacterial agents that kill bacteria are more versatile than those that just inhibit growth, as they can be used as short-term therapies [93], against deep-seated [94,95] and immediately life-threatening infections [95], and in immunocompromised and immunosuppressed patients [95,96]. In the absence of confounding factors, bactericidal agents are defined as those causing a ≥99.9% decrease in bacterial viability at concentrations no more than four times the MIC [97]. Most studies indicate alkaloids are

bactericidal [62,81,98-100], though this can be species-dependent for some alkaloids (eg. chelerythrine, prosopilosidine) [53,59]. Squalamine has been shown to be rapidly bactericidal, with MIC levels reducing the viability of Gram-positive and Gram-negative pathogens by ≥99.99% in just 1-2 hours [81].

4.5 Mechanisms of action

Antibacterial mechanism of action (MOA) has been investigated for alkaloids in the indolizidine, isoquinoline, quinolone, agelasine, and polyamine classes. In the indolizidine class, it has been proposed that the alkaloids pergularinine and tylophorinidine act by inhibiting nucleic acid synthesis, as they inhibit the enzyme dihydrofolate reductase in cell-free assays [101].

In the isoquinoline class, two MOAs have been proposed. Studies with the benzophenanthridine and protoberberine isoquinolines suggest these act by perturbing the Z-ring and inhibiting cell division. Supporting evidence includes demonstrations that sanguinarine and berberine (a) bind to FtsZ [102,103], (b) inhibit FtsZ GTPase activity [103], (c) inhibit Z-ring formation [102-104], and (d) induce cell elongation [102,104] without affecting DNA replication, nucleoid segregation, or membrane structure [102] and without inducing the SOS response [104]. Overexpression and underexpression studies also support this mechanism [104]. Researchers working with the phenanthridine isoquinoline ungeremine suggest this alkaloid acts by inhibiting nucleic acid synthesis after observing inhibition of type I topoisomerases in cell-free assays [105].

Naturally occurring quinolone alkaloids lack the 3-carboxyl group which enables synthetic quinolones like the fluroroquinolones to inhibit the type II topoisomerase enzymes [34]. Research with the alkyl methyl quinolones suggests these are respiratory inhibitors, as they reduce O_2 consupmtion in treated bacteria but do not affect ^3H uptake [79].

The agelasines are a class of alkaloids from the *Agelas* marine sponges [64,65]. Overexpression and binding affinity studies with the antimycobacterial alkaloid agelasine D suggest this exerts its antibacterial effect by inhibiting enzyme BCG 3185c, a suspected dioxygenase, thereby disrupting bacterial homeostasis [65].

Lastly, studies with the polyamine alkaloid squalamine suggest it acts by compromising outer membrane and cytoplasmic membrane integrity. Supporting evidence includes demonstration that squalamine (a) penetrates reconstituted LPS monolayers [106], (b) causes depolarization of the cytoplasmic membrane [81], (c) increases bacterial staining with the cell-impermeable nucleic acid dye propidium iodide [106], and (d) causes leakage of cytoplasmic contents [81,106]. The susceptibility of porin-negative, efflux pump overproducing bacteria to squalamine is consistent with this hypothesis [106].

5. Synergistic and antibiotic-resistance modulating activity

5.1 Naturally occurring alkaloids

Some alkaloids have been reported to increase the antibacterial activity of antibiotics, and information on the five most potent combinations is presented in Table 2a. For tetrandrine and tomatidine, this activity has been confirmed as synergistic (not additive) by determining FIC index values [107,108]. Although the reductions in antibiotic MICs are modest compared to other natural products [109], test alkaloids can exert this effect at quite low concentrations (eg. 0.8 to 1.6 µg/mL for squalamine) [110]. It's also worth noting that comparatively few alkaloids have been tested to date and, based on hit ratio, it is thought many active compounds still await discovery [111].

5.2 Semi-synthetic and synthetic alkaloids

In addition to natural alkaloids, various semi-synthetic and synthetic alkaloids have been reported to increase antibiotic activity (Table 2b). This activity has been confirmed as synergistic for INF392, amlodipine, and compound 4e [114,115,118], and occurs at quite low alkaloid concentrations (eg. 0.4 µg/mL for INF 392, 2.5 µg/mL for compound 13). For amlodipine, this synergy has been demonstrated in vivo, intraperitoneal injection of amlodipine and streptomycin protecting mice against *S.* Typhimurium infection [115]. A second beneficial effect noted with some of these compounds is that the emergence of

antibiotic resistance can be inhibited. For example, INF 392 reduces the rate at which ciprofloxacin resistance emerges by 100-fold [114].

5.3 Structure-activity relationships

Various alkaloid classes have been shown to increase antibiotic activity including the indole [111], piperidine [111,119], pyridine [113], quinoline [111,116], ergoline [112], polyamine [110], and steroidal classes [108], as well as acridine-isoquinoline dimers [117], isoquinoline dimers [107], pyrrole-imidazole dimers [118], and pyridine-pyridine-piperidine trimers [120]. For the steroidal alkaloid tomatidine, activity is dependent upon the spiroaminoketal moiety being in the closed configuration [121]. Active compounds in other alkaloid classes lack an identifiable pharmacophore, but are often lipophilic [111], possess an aromatic ring [111,122], and have a centrally located nitrogen atom [111,122]. The absence of a more rigid SAR for these alkaloids may be related to their mechanism of action. Most are thought to effect synergy by inhibiting bacterial efflux pumps, pumps which themselves act on a wide range of structurally unrelated compounds.

5.4 Mechanisms of action

With the exception of squalamine which enhances antibiotic activity by permeabilizing cells [110] and tomatidine whose MOA is unknown [121], most alkaloids act through efflux pump inhibition. This MOA was established through studies with the model efflux pump substrate ethidium bromide (EtBr). EtBr fluoresces when bound to nucleic acid, and active alkaloids reduce the rate at which EtBr-loaded cells lose fluorescence [112,114,117,120]. If it can reach a sufficiently high intracellular concentration EtBr is also antibacterial, and active alkaloids have been shown to reduce EtBr MICs [111,114,123]. For the ergoline alkaloid lysergol, efflux inhibition is thought to occur due to downregulation and inhibition of efflux pump ATPases [112]. Efflux pumps with confirmed susceptibility to alkaloid inhibition include NorA [114], MexAB-OprM, MexCD-OprJ, MexEF-OprN [116], and ABC transporters [112].

6. Attenuation of bacterial pathogenicity

Bacterial pathogenesis is a multi-stage process typically involving bacterial attachment to host skin or mucous membranes, multiplication, evasion of host defences, then toxin production and/or invasion and inflammation [95]. This process is dependent upon numerous virulence factors, expression of which is tightly regulated.

6.1 Disruption of virulence gene regulation

One mechanism by which bacteria regulate virulence is the use of transcriptional regulators sensitive to environmental conditions. In *Vibrio cholerae*, transcriptional regulator ToxT responds to the presence of intestinal fatty acids and bicarbonate by activating the genes encoding cholera toxin and fimbriae [124]. Recent research shows the isoquinoline alkaloid virstatin inhibits production of both these virulence factors, with microarray and mutant studies identifying ToxT as the likely target [125,126]. Virstatin also has a protective effect in vivo, inhibiting intestinal colonization of infant mice when administered during or after *V. cholerae* inoculation [125].

Quorum-sensing (QS) is another regulatory mechanism. Signal molecules (autoinducers) released by bacteria bind to receptors in neighbouring cells, triggering virulence factor expression when a critical population density is reached. QS-inhibitors have been identified in the indole, 1,3,4-oxadiazole, piperidine, and steroidal alkaloid classes (Table 3). For the indoles, undesirable effects like antibiotic antagonism and biofilm stimulation were initially a problem [127], but these activities have been eliminated in a new semi-synthetic derivative, 7-fluoroindole [128].

6.2 Inhibition of sortase

In Gram-positive bacteria, surface proteins like adhesins, internalins, and immune evasion proteins are attached to the cell wall by enzymes called sortases. Recent studies using subcellular assays show several aaptamine [131], isoquinolone [132], pyrrolidine [133], and bisindole imidazole [134] alkaloids inhibit sortase A. Mass spectrometry studies indicate that, for the pyrrolidine alkaloids, enzyme

inhibition occurs due to covalent modification of the active site nucleophile Cys184 [133]. The impact of this sortase inhibition has been demonstrated at the cellular level too. Whole cells of *S. aureus* treated with sub-MIC bromodeoxytopsentin [134] and isoaaptamine [131] exhibit decreased binding to fibronectin, the host cell receptor which allows bacteria to bind to mucous membranes.

6.3 Disruption of fimbriae and other adhesins

Alkaloids can disrupt adhesins via non-sortase-mediated mechanisms too. Sub-MIC levels of the isoquinoline berberine cause *Streptococcus pyogenes* cells to release lipoteichoic acid, reducing their ability to bind fibronectin [135]. Sub-MIC levels of berberine and 2-pyridone alkaloids disrupt *E. coli* fimbriae via a different mechanism. For berberine the MOA underlying inhibition of fimbrial synthesis is not clearly established [136], but the 2-pyridones disrupt the FGS [137,138] and FGL [139] assembly systems by binding to periplasmic chaperones. Whole-cell studies confirm this results in inhibition of fimbria-dependent biofilm formation in *E. coli* [137].

6.4 Inhibition of bacterial defences against host immune system

Disruption of sortase-dependent immune evasion proteins (eg. protein A) is not the only mechanism by which alkaloids increase bacterial susceptibility to the host immune system. The thiazole alkaloid D157070 (a prodrug for D155931) has been shown to inhibit dihydrolipoamide acyltransferase, the enzyme *M. tuberculosis* uses to detoxify reactive nitrogen intermediates in host macrophages. Whole-cell studies with mycobacteria confirm D157070 increases their sensitivity to nitrite, and promotes killing of cells in infected bone marrow-derived mouse macrophages [140].

6.5 Inhibition of secretion systems

Bacteria use secretion systems to assemble surface structures like adhesins, and export toxins and destructive enzymes. High throughput screening has identified TTS29, a 2-imino-5-arylidene thiazolidinone which inhibits Gram-negative type II and type III secretion systems. The thiazole has

broad-spectrum activity, inhibiting secretion in multiple bacterial species, and protects macrophages against *S.* Typhimurium cytotoxicity. Based on its activity profile, and molecular studies to rule out interference with transcription or translation, the alkaloid is thought to target secretin, an outer membrane protein conserved across the type II and type III systems [141]. Monomeric [141] and dimeric [142] analogues of TTS29 have been produced with enhanced solubility and increased activity.

6.6 Inhibition of exotoxin-mediated effects

Toxins are one of the main mechanisms by which bacteria cause disease. In vivo research with the isoquinoline berberine shows this counteracts *V. cholerae* cholera toxin and *E. coli* heat-stable enterotoxin. Studies were performed with the toxins not the bacterial cells, so the protective effect cannot be related to inhibition of virulence gene regulation, bacterial attachment, or toxin secretion. Given these enterotoxins differ in their MOA, and berberine neither inhibits adenylate cyclase nor enhances intestinal absorption, it is thought the alkaloid acts at a biochemical step after cyclase activation [143].

6.7 Inhibition of destructive enzyme-mediated effects

Another major mechanism by which bacteria cause disease is invasion and inflammation. Bacteria produce destructive proteolytic and glycolytic enzymes, enabling them to breach host tissue barriers and spread to deeper tissue. Whole-cell studies show berberine inhibits bacterial hydrolysis of the connective tissue component collagen [144]. It remains to be established if this is due to inhibition of collagenase production, secretion, or enzymatic action, or an indirect consequence of the alkaloid's antibacterial activity.

6.8 Inhibition of biofilm formation

Biofilm formation protects bacteria from antibiotic therapy, thereby prolonging infections. Numerous alkaloids inhibit the formation of (and/or disperse) bacterial biofilms including imidazoles [145], isoquinolines [100], piperidines [146], pyrrolidines [147], pyrrole-imidazoles [118], and cinchona

alkaloids [148]. In some cases, this inhibition has been attributed to direct antibacterial activity [148], in others to QS disruption [146,147], or unknown causes [100,118], possibly inhibition of sortase or adhesins.

7. Concluding remarks

Alkaloids have a proven track record as drug scaffolds and scaffold substructures in modern antibacterial chemotherapy [39]. This review highlights other antibacterial alkaloids with development potential, the quinolone CJ-13,136, for example, with MICs as low as 0.1 ng/mL against *H. pylori*, and the polyamine alkaloid squalamine reducing ciprofloxacin MICs ≥16-fold against *Klebsiella pneumoniae*. For many of these compounds, further characterization is necessary eg. determination of spectrum of activity, MIC_{90} values (ie. MIC required to inhibit 90% of strains of a species), FIC index values, MOA, toxicity, SAR, solubility and stability, resistance frequency, and serum protein binding. In terms of MOA, it has been proposed, based on assays with purified enzymes, that indolizidine alkaloids inhibit dihydrofolate reductase, and isoquinoline alkaloids inhibit type I topoisomerases. Whole-cell studies (eg. target overexpression, underexpression, and alteration) would help rule out nonspecific inhibition and confirm these MOAs. Selectivity also needs to be established as some antibacterial (eg. manzamine A [149]) and antibiotic-enhancing (eg. reserpine [114,120]) alkaloids are highly toxic to eukaryotic cells. Because some alkaloids are immunosuppressive (eg. gliotoxin [150]), toxicity testing should evaluate this parameter too.

In addition to direct antibacterial and antibiotic-enhancing activities, alkaloid inhibition of bacterial virulence has been described. For alkaloids like berberine which exert both direct antibacterial and antivirulence effects, the implication is that these will, like the QS-inhibiting macrolide antibiotics [151], exert greater in vivo efficacy than their MIC evaluations suggest. For alkaloids that inhibit bacterial virulence without affecting growth or viability, these could potentially be developed as antivirulence drugs. A common concern with antivirulence drugs is that narrow-spectrum activity could preclude empiric use. Until real-time diagnostics are more widely available, efforts should therefore

focus on alkaloids likely to have broad-spectrum activity ie. inhibitors of secretion systems [141] and other virulence factors conserved across bacterial species. A second concern with antivirulence drugs is that, because they do not kill bacteria, they could not be used in immunocompromised patients. It may be possible to overcome this problem by targeting virulence factors required for colonization and maintenance of attachment (eg. adhesins and adhesin synthesising machinery) rather than disease symptoms (eg. toxins).

Regarding future discovery and development efforts, there are opportunities to expedite this process. By departing from what, in academia, is often a monodisciplinary approach, and promoting greater collaboration between chemists, microbiologists, and pharmacologists, it should be possible for more groups to isolate active compounds and generate robust microbiological and toxicological data. Given the risk of toxicity with alkaloids, tests for overt toxicity should be performed at the earliest opportunity. Where cell culture is available, non-malignant cell lines should be tested. In more resource-limited settings, tests like the brine shrimp lethality assay or haemolysis assay could be used. Lastly, greater use should be made of the many new techniques and technologies available eg. efflux pump mutants to detect efflux-susceptible antibacterial agents [152], synergy-directed fractionation to detect natural product components that work synergistically [153], medicinal chemistry techniques to conjugate efflux susceptible antibacterials with efflux pump inhibitors [86], and genetic techniques [104] and reporter strains of bacteria [154] for MOA elucidation.

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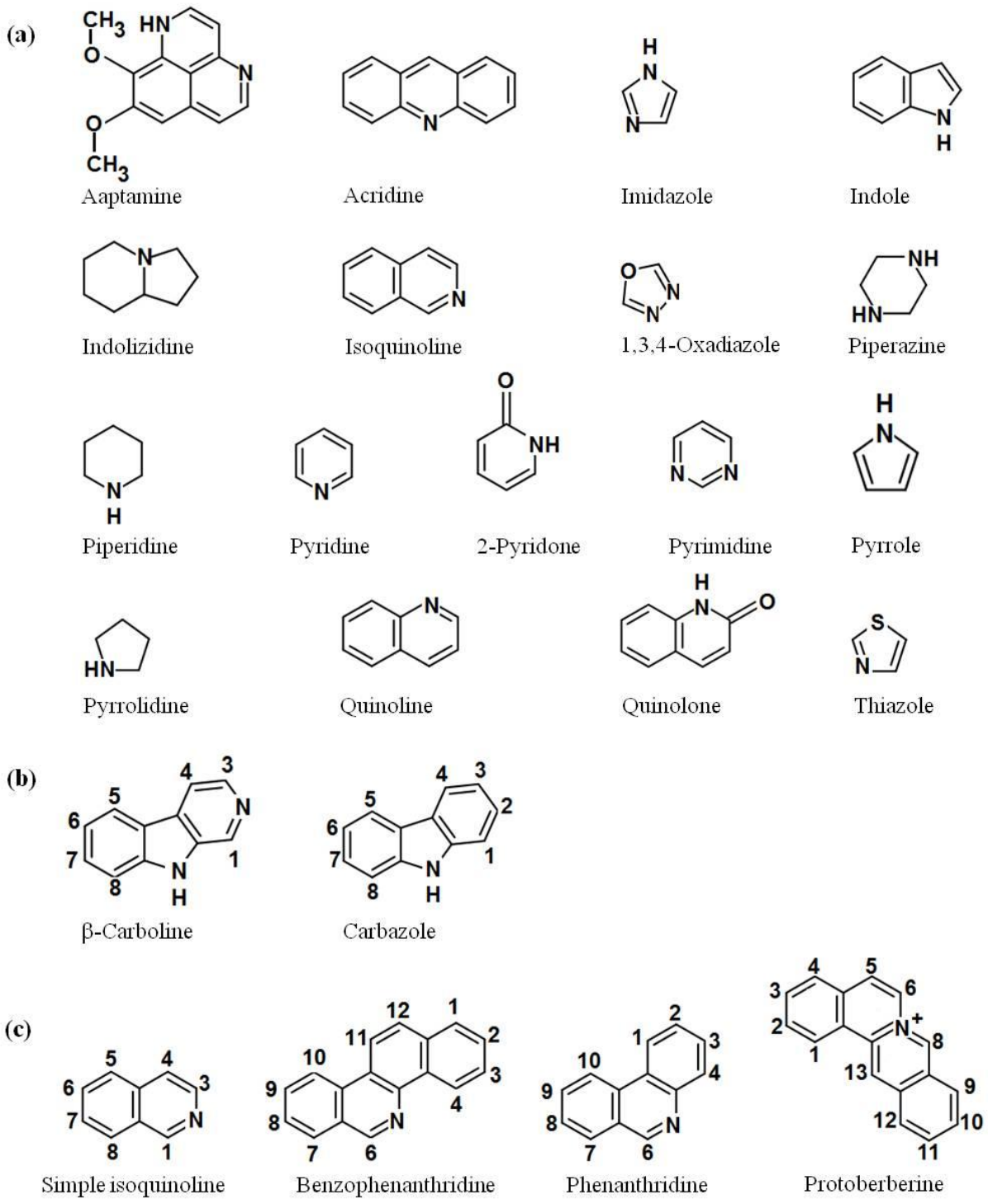

Figure 1 Skeleton structures of (a) the major heterocyclic alkaloid classes possessing antibacterial activity and important antibacterial subclasses of the (b) indole and (c) isoquinoline alkaloids.

Table 1 Information on the ten most potently antibacterial natural alkaloids as identified by PubMed and ScienceDirect searches (no date restrictions).

Alkaloid	Alkaloid class (& sub-class)	MIC assay	Cell density (CFU/mL)	MIC (μg/mL)			Reference
				Gram positive	Gram negative	Mycobacteria	
CJ-13,136	Quinolone	BMAD	1×10^5	NA	0.0001	NT	[63]*
Ascididemin	Pyridoacridine	BMID	2×10^8	0.08	0.06	NT	[71]*
Xinghaiamine A	Miscellaneous	BMID	1×10^5	0.3 to 4	0.1 to 2	NT	[80]
Eudistomin Y_4	Indole (β-carboline)	BMID	5×10^5	0.8 to 3.1	0.4 to 50	NT	[51]*
Hapalindole I	Indole	MABA	1×10^5	NA	NA	0.7	[52]*
Squalamine	Polyamine	BMID	1×10^5	1 to 2	1 to >100	NT	[32,81,82]
Prosopilosidine	Indolizidine	BMID	5×10^5	1.3	NT	2.5	[53]*
Clausenol	Indole (carbazole)	AD	NS	1.3 to 14	7 to 14	NT	[47]
Chelerythrine	Isoquinoline (benzophenanthridine)	BMID	1×10^5	1.5	1.5	NT	[59]*
8-ADHN	Isoquinoline	NS	NS	1.6	NA	NT	[56]*
Agelasine D	Agelasine	MTT	1×10^5	NT	NT	1.6 to 3.1	[65]
Sanguinarine	Isoquinoline (benzophenanthridine)	BMID	1×10^6	1.6 to 6.3	NT	NT	[58]

Notes: MIC ranges listed are for human pathogens. Where MIC protocols were not stated or ambiguous in the journal article, authors were contacted for further details. 8-ADHN (8-acetonyldihydronitidine), agelasine D, and sanguinarine have comparable levels of antibacterial activity, so all three have been included in the table. Studies marked with an asterisk identified more than one highly active compound. BMAD, broth macrodilution assay; BMID, broth microdilution assay; MABA, microplate Alamar Blue assay; MTT, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay; NS, not stated; NT, not tested; NA, not active (MIC >100μg/mL)

Table 2 Information on the five most potently synergistic alkaloid-antibiotic combinations as identified by PubMed and ScienceDirect searches (no date restrictions).

(a) Naturally occurring alkaloids

Alkaloid	Alkaloid class	Antibiotic	Test bacteria	Reduction in antibiotic MIC	Reference
Squalamine	Polyamine	Ciprofloxacin	*Klebsiella pneumoniae* [a]	16- to >32-fold	[110]*
Lysergol	Ergoline	Tetracycline	*Escherichia coli* [b]	8-fold	[112]*
Tetrandrine	Bisisoquinoline	Cefazolin	MRSA	4-fold	[107]
Compound 8	Pyridine	Ciprofloxacin	*S. aureus* [c]	3-fold	[113]*
Tomatidine	Steroidal	Gentamycin	*S. aureus* [d]	2- to 32-fold	[108]*

(b) Semi-synthetic and synthetic alkaloids

Alkaloid	Alkaloid class	Antibiotic(s)	Test bacteria	Reduction in antibiotic MIC	Reference
INF392	Pyrimidine	Ciprofloxacin	*Bacillus subtilis* [c]	8-fold	[114]
Amlodipine	Piperidine	Streptomycin	*Salmonella* Typhimurium	6.5- to 8-fold	[115]
Compound 13	Quinoline	Levofloxacin	*P. aeruginosa* [a]	4- to 8-fold	[116]
GG918	Acridine-Isoquinoline	Norfloxacin	*S. aureus* [a]	4- to 8-fold	[117]*
Compound 4e	Pyrrole-Imidazole	Oxacillin	MRSA	4-fold	[118]

Notes: All of the above studies tested for synergy using the broth microdilution method and/or checkerboard test. Semi-synthetic alkaloids which showed no improvement in activity compared to their parent structure have been excluded. Studies marked with an asterisk detected synergy with more than one antibiotic and/or against more than one species. a, efflux pump overproducing; b, multidrug resistant; c, NorA (efflux pump) expressing; d, antibiotic-sensitive and multidrug resistant

Table 3 Alkaloids identified as QS inhibitors based on their effects upon QS-regulated virulence factors and processes, and / or their interactions with specific QS targets.

Alkaloid (& alkaloid class)	Virulence factors / processes inhibited	QS target(s)	Reference
Compound 37 (1,3,4-Oxadiazole)	Inhibits toxin (pyocyanin) & QS signal precursor (HHQ) production in *P. aeruginosa*	PqsR	[129]
7-Hydroxyindole (Indole)	Alters virulence gene expression, inhibits toxin (pyocyanin), QS signal (PQS), biosurfactant (rhamnolipid), & siderophore (pyochelin) production, & abolishes swarming motility in *P. aeruginosa*	PQS, PqsR	[127]
Solenopsin A (Piperidine)	Inhibits virulence gene transcription, toxin (pyocyanin) & destructive enzyme (elastase B) production, & biofilm formation in *P. aeruginosa*	*rhl* system	[130]
Tomatidine (Steroidal)	Inhibits virulence gene expression & haemolytic activity in *S. aureus*	*agr* system	[108]

QS, quorum sensing; PQS, *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4(1*H*)-quinolone); PqsR, PQS transcriptional regulator (also known as 'Multiple virulence factor regulator' or 'MvfR'); HHQ, 2-heptyl-4-(1H)-quinolone (a precursor of PQS)

Supplementary Table 1 Details of alkaloids discussed within the review.

(a) Alkaloid monomers

Alkaloid class	Alkaloid		ChemSpider ID	Molecular formula	Reference
	Published name	Systematic name			
Aaptamine	Isoaaptamine	8-Methoxy-1-methyl-1H-benzo[de][1,6]naphthyridin-9-ol	350474	$C_{13}H_{12}N_2O_2$	[1]
Agelasine	Agelasine D	6-Amino-9-methyl-7-[(2E)-3-methyl-5-(5,5,8a-trimethyl-2-methylenedecahydro-1-naphthalenyl)-2-penten-1-yl]-7H-purin-9-ium chloride	28580684	$C_{26}H_{40}ClN_5$	[2]
Ergoline	Lysergol	[(8β)-6-Methyl-9,10-didehydroergolin-8-yl]methanol	14267	$C_{16}H_{18}N_2O$	[3]
Indole	Clausenol	6-Methoxy-3-methyl-9H-carbazol-1-ol	8529137	$C_{14}H_{13}NO_2$	[4]
	7-Fluoroindole	7-Fluoro-1H-indole	2054901	C_8H_6FN	[5]
	7-Hydroxyindole	1H-Indol-4-ol	67953	C_8H_7NO	[6]
	Eudistomin Y_4	(6-Bromo-9H-β-carbolin-1-yl)(3-bromo-4-hydroxyphenyl)methanone	23314571	$C_{18}H_{10}Br_2N_2O_2$	[7]
	Hapalindole I	(6aR,8R,9R)-8-Chloro-10-isocyano-6,6,9-trimethyl-9-vinyl-2,6,6a,7,8,9-hexahydronaphtho[1,2,3-cd]indole	10475139	$C_{21}H_{21}Cl\ N_2$	[8]
Indolizidine	Pergularinine	(13aS,14R)-3,6,7-Trimethoxy-9,11,12,13,13a,14-hexahydrodibenzo[f,h]pyrrolo[1,2-b]isoquinolin-14-ol	232616	$C_{23}H_{25}NO_4$	[9]
	Prosopilosidine	6-{10-[(2R,5R,6S)-5-Hydroxy-6-methyl-2-piperidinyl]decyl}-8-{10-[(2R,5S,6R)-5-hydroxy-6-methyl-2-piperidinyl]decyl}-2,3-dihydro-1H-indolizinium chloride	25061528	$C_{40}H_{72}ClN_3O_2$	[10]
	Tylophoridinine	(13aS,14S)-3,7-Dimethoxy-9,11,12,13,13a,14-hexahydrodibenzo[f,h]pyrrolo[1,2-b]isoquinoline-6,14-diol	142063	$C_{22}H_{23}NO_4$	[9]
Isoquinoline	8-Acetonyldihydronitidine (8-ADHN)	1-(2,3-Dimethoxy-12-methyl-12,13-dihydro[1,3]benzodioxolo[5,6-c]phenanthridin-13-yl)acetone	8915378	$C_{24}H_{23}NO_5$	[11]
	Berberine	9,10-Dimethoxy-5,6-dihydro[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium	2263	$C_{20}H_{18}NO_4$	[12]

Alkaloid class	Alkaloid		ChemSpider ID	Molecular formula	Reference
	Published name	Systematic name			
Isoquinoline (ctd.)	Chelerythrine	1,2-Dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridin-12-ium	2602	$C_{21}H_{18}NO_4$	[13]
	Compound 9	1-(4-Biphenylyl)-2,3,7,8-tetramethoxy-5-methylbenzo[c]phenanthridinium	28651733	$C_{34}H_{30}NO_4$	[14]
	Sanguinarine	13-Methyl[1,3]benzodioxolo[5,6-c][1,3]dioxolo[4,5-i]phenanthridin-13-ium	4970	$C_{20}H_{14}NO_4$	[15]
	SS14	9,10-Dimethoxy-13-[2-(5-nitro-1H-indol-2-yl)benzyl]-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-α]quinolizinium bromide	-	$C_{35}H_{28}N_3O_6$	[16]
	Ungeremine	2-Hydroxy-4,5-dihydro[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridin-6-ium	140368	$C_{16}H_{12}NO_3$	[17]
	Virstatin	4-(1,3-Dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)butanoic acid	128749	$C_{16}H_{13}NO_4$	[18]
Manzamine	8-*n*-hexamidomanzamine A	N-{1-[(1R,2R,4R,5Z,12R,13S,16Z)-13-Hydroxy-11,22-diazapentacyclo [11.11.2.1^{2,22}.0^{2,12}.0^{4,11}]heptacosa-5,16,25-trien-25-yl]-9H-β-carbolin-8-yl}hexanamide	24647972	$C_{42}H_{55}N_5O_2$	[19]
1,3,4-Oxadiazole	Compound 37	5-[3-(Trifluoromethyl)phenyl]-1,3,4-oxadiazol-2-amine	20618	$C_9H_6F_3N_3O$	[20]
Piperidine	Amlodipine	3-Ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydro-3,5-pyridinedicarboxylate	2077	$C_{20}H_{25}ClN_2O_5$	[21]
	Solenopsin A	(2R,6R)-2-Methyl-6-undecylpiperidine	65431	$C_{17}H_{35}N$	[22]
Polyamine	Squalamine	(3β,5α,7α,24R)-3-({3-[(4-Aminobutyl)amino]propyl}amino)-7-hydroxycholestan-24-yl hydrogen sulfate	65407	$C_{34}H_{65}N_3O_5S$	[23]
Pyridine	Compound 8	Diethyl 2,6-dimethyl-4-phenyl-3,5-pyridinedicarboxylate	547037	$C_{19}H_{21}NO_4$	[24]
Pyrimidine	INF392	2-(Benzylthio)-5-(diphenylmethyl)-6-hydroxy-4(1H)-pyrimidinone	-	$C_{24}H_{20}N_2O_2S$	[25]
Pyrrole	Compound 4m	4,5-Dibromo-N'-(4-(2,6-dichlorophenoxy)benzylidene)-1-methyl-1H-pyrrole-2-carbohydrazide	-	Not stated	[26]
Quinoline	Compound 13	N^2-[(2S)-2-Amino-4-phenylbutanoyl]-N-3-quinolinyl-L-ornithinamide	419715	$C_{24}H_{29}N_5O_2$	[27]
Quinolone	CJ-13,136	2-[(2E)-3,7-Dimethyl-2,6-octadien-1-yl]-3-methyl-4(1H)-quinolinone	8079894	$C_{20}H_{25}NO$	[28]

Alkaloid class	Alkaloid		ChemSpider ID	Molecular formula	Reference
	Published name	Systematic name			
Quinolone (ctd.)	Not stated	1-Methyl-2[(Z)-7-tridecenyl]-4-(1H)-quinolone	-	$C_{23}H_{33}NO$	[29]
	Evocarpine	1-Methyl-2[(Z)-8-tridecenyl]-4-(1H)-quinolone	4524183	$C_{23}H_{33}NO$	[29]
Steroidal	Tomatidine	(3β,5α,25S)-Spirosolan-3-ol	59019	$C_{27}H_{45}NO_2$	[30]
Thiazole	CCR-11	2-Thioxo-5-({5-[3-(trifluoromethyl)phenyl]-2-furyl}methylene)-1,3-thiazolidin-4-one	2120791	$C_{15}H_8F_3NO_2S_2$	[31]
	D155931	3-[(5Z)-5-{[5-(2-Chlorophenyl)-2-furyl]methylene}-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]benzoic acid	24958530	$C_{21}H_{12}ClNO_4S_2$	[32]
	D157070	3-Hydroxypropyl 3-[(5Z)-5-{[5-(2-chlorophenyl)-2-furyl]methylene}-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]benzoate	24958531	$C_{24}H_{18}ClNO_5S_2$	[32]
	TTS29	(2Z,5Z)-5-(4-Hydroxy-3,5-dimethoxybenzylidene)-3-phenyl-2-(phenylimino)-1,3-thiazolidin-4-one	1050113	$C_{24}H_{20}N_2O_4S$	[33]
Miscellaneous	Xinghaiamine A	Not stated	-	$C_{50}H_{48}N_2OSNa$	[34]

(b) Alkaloid dimers and oligomers

Alkaloid class	Alkaloid		ChemSpider ID	Molecular formula	Reference
	Published name	Systematic name			
Acridine-Isoquinoline	GG918	N-{4-[2-(6,7-Dimethoxy-3,4-dihydro-2(1H)-isoquinolinyl)ethyl]phenyl}-5-methoxy-9-oxo-9,10-dihydro-4-acridinecarboxamide	106620	$C_{34}H_{33}N_3O_5$	[35]
Bisindole	NCD9	2,2'-(1,10-Decanediyl)bis(6-chloro-9H-β-carbolin-2-ium)	25036916	$C_{32}H_{34}Cl_2N_4$	[36]
	5,6,6'-Tribromo-1H,1'H-2,2'-biindole	Not stated	-	$C_{16}H_9Br_3N_2$	[37]
Bisindole-Imidazole	Bromodeoxytopsentin	[5-(6-Bromo-1H-indol-3-yl)-1H-imidazol-2-yl](1H-indol-3-yl)methanone	354909	$C_{20}H_{13}BrN_4O$	[38]

Alkaloid class	Alkaloid		ChemSpider ID	Molecular formula	Reference
	Published name	Systematic name			
Bisisoquinoline	Tetrandrine	(1β,1'ξ)-6,6',7,12-Tetramethoxy-2,2'-dimethylberbaman	4479515	$C_{38}H_{42}N_2O_6$	[39]
Pyridoacridine	Ascididemin	9H-Quinolino[4,3,2-de][1,10]phenanthrolin-9-one	164401	$C_{18}H_9N_3O$	[40]
Pyrrole-Imidazole	Compound 4e	N-(2-{4-[5-(2-Amino-1-isobutyl-1H-imidazol-4-yl)pentyl]-1H-1,2,3-triazol-1-yl}ethyl)-4-pentylbenzamide hydrochloride (1:1)	29417820	$C_{28}H_{44}ClN_7O$	[41]
Bisthiazole	Compound 6f	2-((5*Z*)-5-((2-(trifluoromethyl)-6-(4-methoxyphenyl) imidazo [2,1-*b*][1,3,4]-thiadiazol-5-yl)methylene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid	-	$C_{18}H_{11}F_3N_4O_4S_3$	[42]

Note: Readers are referred to the Royal Society of Chemistry ChemSpider database (http://www.chemspider.com/) and supporting references for alkaloid structures and further details. -, not available

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