Solid Phase Synthesis of Fumitremorgin-type Alkaloids based on Cyclization/Cleavage Strategy

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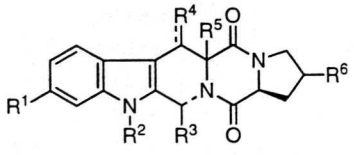
Chapter 2

Fumitremorgin-type Indole Alkaloids

Abstract

Indole alkaloids of the fumitremorgin/verruculogen/(cyclo)tryprostatin class have been isolated from various fungal sources, mainly *Aspergillus* and *Penicillium* strains. The (bio)synthesis of these, as well as other prenylated indolyl diketopiperazine alkaloids, has attracted a lot of interest. This is not only due to their often intriguing chemical structure, but also as a result of the interesting biological activities that frequently provoked their isolation.

The basis for the discovery of fumitremorgin-type indolyl diketopiperazines in the 1970's was their ability to induce tremors in vertebrates, due to interference with neurotransmitter release in the CNS. In 1995, members of this compound class were shown to inhibit cell cycle progression at the G2/M phase; interference in the process of microtubule assembly proved to be the main mechanism of action. In 1998, fumitremorgin C was found to be a potent and selective inhibitor of the newly discovered ABC transporter BCRP: a drug efflux pump part of an organism's natural defense mechanism against xenobiotics, that is overexpressed in certain multidrug resistant cancer cells.



R^1 = —H , —OMe

R^2 = —H , $—CH_2-CH=C(CH_3)_2$

R^3 = $—CH=C(CH_3)_2$, $—CH_2-C(CH_3)_2-OH$

R^4 = —H , ····OH , ····OMe , =O , ····$O-CH_2-CH=C(CH_3)_2$

R^5 = ····H , ····OH , —OH

R^6 = —H , —OAc

2.1 Introduction

2.1.1 *Indole alkaloids*

Due to their often intriguing chemical structure and interesting biological activity, natural products –in particular secondary metabolites– have always been a source of inspiration for synthetic and medicinal chemists. Many of these biologically active compounds are alkaloids isolated from marine, fungal and botanical organisms. They are frequently believed to play a role in the survival strategy of the species, for instance as 'chemical' defense mechanism against other organisms, which may explain the wide variety of biological activities found among alkaloids originating from different species.

Among the bioactive alkaloids, those containing an indole moiety form a major class. Because of their structural relationship to the essential amino acid tryptophan, and its corresponding metabolites such as the neurotransmitter serotonine (5-hydroxytryptamine, 5-HT), indole alkaloids have acquired a prominent position in natural product chemistry. Frequently, the indole structural element is part of a tricyclic (tetra- or dihydro-) β-carboline unit, or in the form of a pyrrolo-indole. Many indole alkaloids can be biosynthetically dissected to an indole component formally derived from L-tryptophan, and other building blocks derived from different amino acids (non-terpenoid indole alkaloids) or the branched isoprene skeleton (terpene derived indole alkaloids). The use of these natural building blocks or metabolites thereof, and the potential of different biogenetical transformations and oxidation or reduction steps during biosynthesis, provides a wide variety of structurally different indole alkaloids.

2.1.2 *Discovery of fumitremorgin-type indolyl diketopiperazines*

A class of indole alkaloids that combines a tryptophan-derived dipeptide moiety and one or more isoprene-derived units is that of the fumitremorgins, verruculogens and tryprostatins. Their common skeleton is derived from an L-tryptophan-L-proline diketopiperazine condensed to a tetrahydro-β-carboline system with an isoprene unit. They form intriguing systems since both tetrahydro-β-carbolines and diketopiperazines are frequently seen as pharmacophoric core structures. In this light, it is not surprising that the compounds of this class were often discovered in screening programs for isolation and identification of biologically active secondary metabolites from natural sources.

In 1964, screening fungus cultures growing on foodstuffs, including various strains of *Aspergillus*, revealed the existence of a previously unknown secondary metabolite in *Aspergillus flavus* capable of inducing sustained tremors (a compound property rarely observed[1]) in mice, guinea pigs and rats, sometimes followed by convulsions, depending upon the dosage.[2] Though the response to this new compound was somewhat similar to that elicited by tremorine (1,4-dipyrrolidino-2-butyne), this compound differed in several

significant respects. Four years later, in an investigation on the cause of outbreaks of disease among farm animals, a composite of an isolate from *Penicillium cyclopium* showed similar neurotoxic properties.[3] An identically behaving mycotoxin was obtained from *Penicillium palitans*, a mold closely related to *Penicillium cyclopium*.[4] These same and also new, similar biologically active compounds were obtained from other *Penicillium* strains,[5],[6] all containing an indole moiety providing the sole nitrogen atom in the molecule.

In 1971, two compounds having strong tremorgenic action in mice were isolated from *Aspergillus fumigatus*, and named fumitremorgin A (**1**) and B (**2**) (Figure 2.2).[7] UV and NMR spectra of both compounds suggested the presence of a 2,3-disubstituted 6-methoxyindole system. The presence of an indole moiety was supported by culture experiments in the absence and presence of L-tryptophan in the medium, and incorporation of radioactivity from ^{14}C-labeled tryptophan. One year later, a new tremorgenic mycotoxin named verruculogen (also known as TR-1) (**5**) was obtained from a strain of *Penicillium verruculosum*.[8] UV analysis revealed the presence of an indole system, whereas the IR spectrum suggested amide functionality and possibly a *gem*-dimethyl group. In a further structure elucidation study,[9] verruculogen TR-1 (**5**) proved to be closely related to the fumitremorgins in having a 6-methoxy substituent on the indole unit (which is absent in the other *Penicillium* tremorgens) and in containing three nitrogen atoms per molecule. In addition, proof for the presence of a β-methylcrotonyl moiety was presented.

The first structure to be established, though not to detailed stereochemical extent, was that of fumitremorgin B (**2**),[10] soon to be followed by that of verruculogen TR-1 (**5**)[11] and the still tremorgenic hydrogenation product of the latter, verruculogen TR-2 (**7**).[12] Verruculogen TR-2 (**7**) was later also isolated from *Aspergillus fumigatus*[13] and shown to be an intermediate in the biosynthesis of verruculogen TR-1 (**5**) and fumitremorgin B (**2**).[14] In 1975, the crystal structure and thus the absolute configuration of fumitremorgin B (**2**) were published,[15] and the structure determination[16] was extensively discussed in a following article.[17] In the same year the structure of fumitremorgin A (**1**), previously isolated but not characterized,[10] was reported independently by two groups.[18],[19] Both fumitremorgin A (**1**) and verruculogen TR-1 (**5**) possess an unusual peroxide link between two isoprene units. Though this peroxide functionality plausibly arises from the hydroperoxide, this intermediate has not been detected. A detailed study of the biosynthesis of verruculogen TR-1 (**7**), with a focus on the origin of the oxygen atoms and the stereochemical course of the peroxide ring formation, has been published.[20] Ironically, the structure of the most simple member of the compound class, fumitremorgin C (**3**), was only reported in 1981,[21] though its isolation as a compound called SM-Q was already mentioned in 1977.[13]

All these compounds were shown to be based on an L-tryptophan-L-proline diketopiperazine skeleton ring fused with an isoprenyl-derived unit evolving from mevalonate to form a tetrahydro-β-carboline type pentacyclic structure. Other isoprenyl units as well as a 6-methoxy substituent on the indole that is originating from methionine may be present in compounds of this class (Figure 2.1).[22]

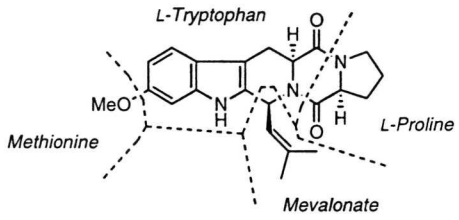


Figure 2.1: *Bioprecursors of the regions of the fumitremorgin framework.*

A 1982 report revealed the natural existence of acetoxy-verruculogen (**6**) possessing an acetylated 4-hydroxyproline moiety.[23] The first synthesis of 6-demethoxy-fumitremorgin C (**4**) was published in 1987,[24] whereas in 1996 it was also isolated from natural sources.[25] Another secondary metabolite was isolated in 1990 and was structurally designated as 12,13-dihydroxyfumitremorgin C (**8**)[26] (Figure 2.2).

In 1995, two new members of the fumitremorgin-type compound class were isolated from a marine fungal strain BM939, identified as *Aspergillus fumigatus*, in which the central ring of the system is in its open form and the isoprenyl unit is connected to the indole 2-position.[27],[28] They were isolated in the course of a screening for new mammalian cell cycle inhibitors from microbial origin, in which fumitremorgin C (**3**) and demethoxy-fumitremorgin C (**4**) later also proved to be active.[29] The ring-opened analog of fumitremorgin C (**3**) was named tryprostatin A (**9**), whereas the ring-opened demethoxy-fumitremorgin C (**4**) analog was called tryprostatin B (**10**). The cyclotryprostatins A (**11**), B (**12**), C (**13**) and D (**14**), novel cell cycle inhibitors reported in 1997,[30] again are pentacyclic fumitremorgin analogs.

2.1.3 *Other indolyl diketopiperazine based alkaloids*

Besides the discovery of secondary metabolites of the fumitremorgin-type, many other alkaloids have been discovered uniting an indole and a diketopiperazine moiety, and often one or more isoprenyl units. In 1973, five diketopiperazines were isolated from strains of *Aspergillus Ustus* and found to cause acute toxicosis in day-old ducklings.[31] Two of the components showed to be the previously reported[32] austamide (**18**) and its dihydro-derivative (**19**) (Figure 2.3). Both compounds are ψ-indoxyl alkaloids in which a 'reverse prenyl' (1,1-dimethyl-2-propenyl) unit connects the indoxyl 2-position to a diketopiperazine nitrogen. The corresponding indole derivative of (**18**), alkaloid (**20**), was isolated as well, together with two other indole alkaloids with a reversed prenyl substituent on the indole 2-position: deoxybrevianamide E (**16**) and its didehydro analog (**17**). Formally, the trivial name of (**16**), deoxybrevianamide E, is incorrect, as it does not involve the mere loss of one oxygen atom from brevianamide E (**15**), a pyrrolo-indole (hexahydropyrrolo-[2.3-*b*]-indole) alkaloid described earlier.[33] The same type of reversed linkage of the isoprenoid unit to the indole 2-position is found in echinulin (**23**),[34] which possesses an L-tryptophan-L-alanine diketopiperazine framework, instead of the L-tryptophan-L-proline skeleton in the alkaloids

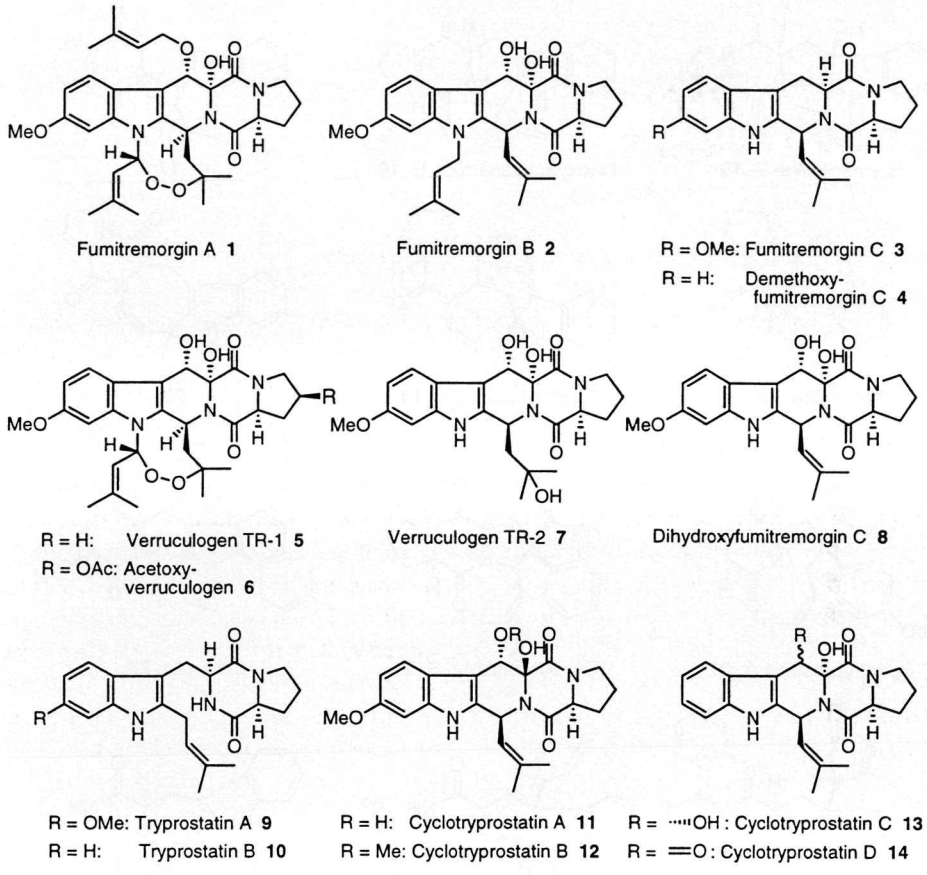
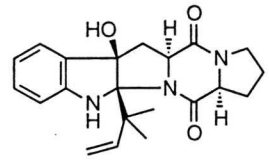


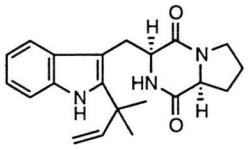
Figure 2.2: *Members of the fumitremorgin / verruculogen / tryprostatin class.*

described above. The rather unusual isoprenoid orientation presumably arises from 'normal' alkylation of the indole nitrogen (N-1) and a subsequent cyclic rearrangement (Scheme 2.1, pathway A). [35],[36] Although initial C-3 alkylation could theoretically give the same result (Scheme 2.1, pathway B), investigations have revealed that this is a less likely process.[37]

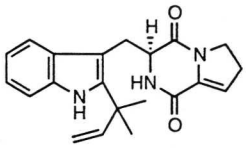
Amauromine (**31**) is a C_2-symmetrical di-pyrrolo-indolyl diketopiperazine possessing two reversed prenyl substituents on de former indole 3-positions.[38] This alkaloid was isolated from *Amauroascus sp.* and shows potent vasodilating activity[39] originating from calcium antagonism.[40] Its full structure elucidation was described in 1985.[41] Structurally closely related is roquefortine C (**32**),[42] and its dihydro derivative roquefortine D,[43] isolated from the cultures of *Penicillium roqueforti*. In these molecules, a histidine unit is one of the incorporated bioprecursors.[44] These C-3 reverse prenyl indole alkaloids conceivably originate biosynthetically from S_N2'-type alkylation *via* a prenylated cysteine of a prenyl transferase, which in turn may be generated *via* the action of prenyl pyrophosphate on a



Brevianamide E **15**



Deoxybrevianamide E **16**



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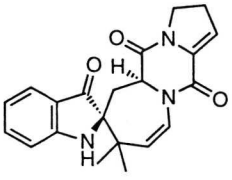
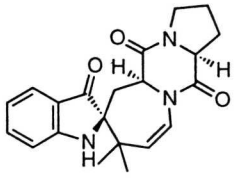
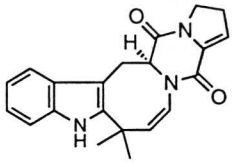


Austamide **18**



Dihydroaustamide **19**



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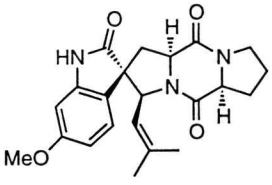
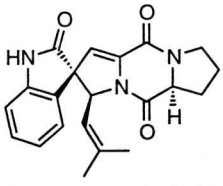
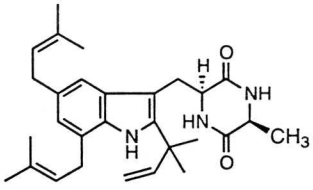


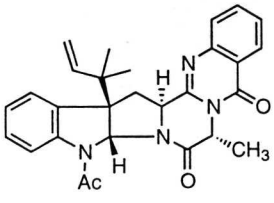
Spirotryprostatin A **21**



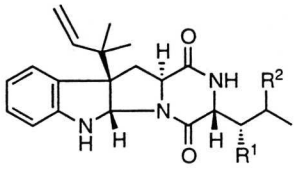
Spirotryprostatin B **22**



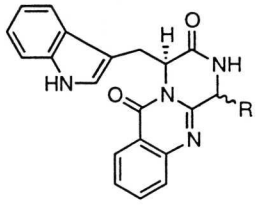
Echinulin **23**



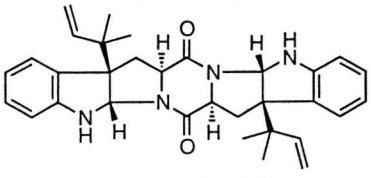
N-acetylardeemin **24**



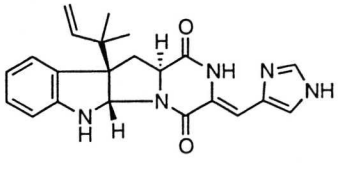
R^1 = Me, R^2 = H:
Brevicompanine A **25**
R^1 = H, R^2 = Me:
Brevicompanine B **26**



R = H: Glyantrypine **27**
R = ━ *i*-Pr: Fiscalin B **28**
R = ━ Me: Fumiquinazoline F **29**
R = ┈ Me: Fumiquinazoline G **30**

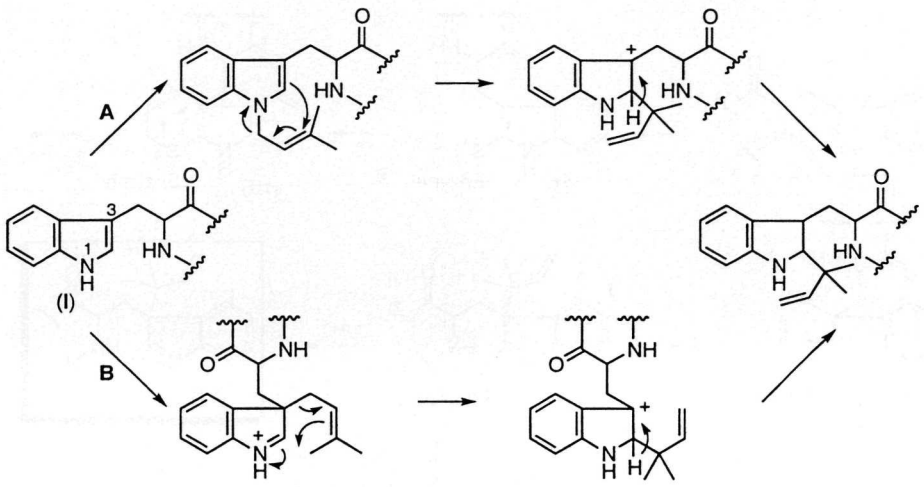


Amauromine **31**



Roquefortine C **32**

Figure 2.3: *Other indolyl diketopiperazine alkaloids.*

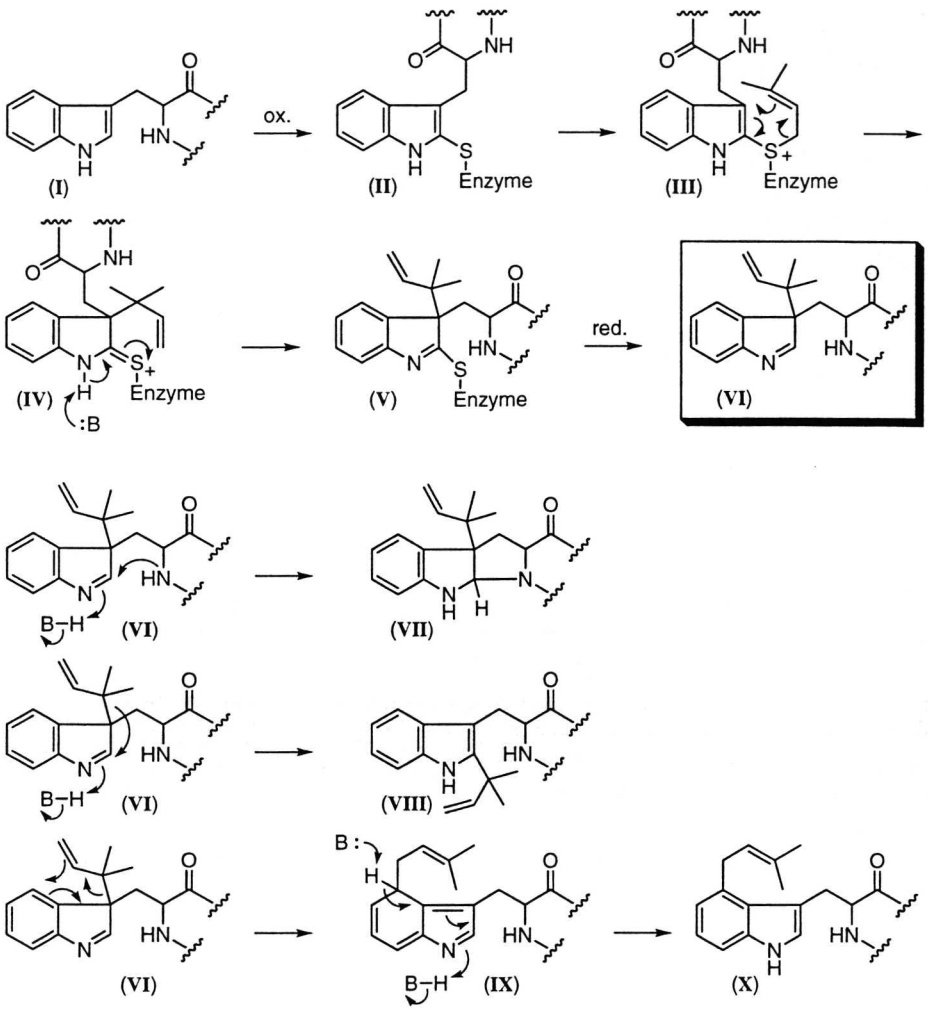


Scheme 2.1: *Putative genesis of reverse prenyl substituents.*

cysteine residue.[45] A thio-Claisen type rearrangement of intermediate (**III**) is the key step in the formation of indolenine structure (**VI**). The pyrrolo-indole construction (**VII**) then would be completed by 5-*exo* cyclization[46] of the tryptophyl α-amino group onto the intermediate indolenine (**VI**) (Scheme 2.2). The latter compound may also account for the formation of other frameworks. A Plancher-type rearrangement[47] of (**VI**) would afford C-2 reverse prenyl substituted indoles (**VIII**), whereas further cyclic rearrangement to (**IX**) may lead to a known precursor (**X**) of the ergot alkaloids.[48]

Though in most diketopiperazine alkaloids the amino acid building blocks are in the natural *S*-configuration, as is also the case for roquefortine D, examples are known in which the sidechains of the non-tryptophan part of the diketopiperazine have the unnatural D-amino acid configuration. For instance two plant growth regulators produced by *Penicillium brevicompactum*, brevicompanines A (**25**) and B (**26**),[49] have incorporated an *allo*-isoleucine or a leucine unit in the *R*-configuration, respectively. The framework corresponding to brevicompanine B (**26**), but with the all-natural amino acid configuration, is found in fructigenine B (leucine residue) and fructigenine A (phenylalanine residue).[50]

In *N*-acetylardeemin (**24**), a fungal metabolite from *Aspergillus fischerii* capable of reversing drug insensitivity in certain tumor cells,[51] a D-alanine building block is part of the architecture.[52] Another structural aspect of (**24**) is that the diketopiperazine unit is fused, through a benzopyrazinone motif, to an anthranilic acid moiety. Though on the opposite side of the diketopiperazine ring, this substructure comes back in the depicted compounds of the glyantrypine/fiscalin/fumiquinazoline (**27-30**) class of alkaloids. Glyantrypine (**27**), containing a glycine residue, was isolated from *Aspergillus clavatus*.[53] Fiscalin B (**28**), a substance P antagonist produced by *Neosartorya fischeri* and *Corynascus setosus*,[54] has a L-valine incorporated, whereas the fumiquinazolines F (**29**) and G (**30**), compounds isolated

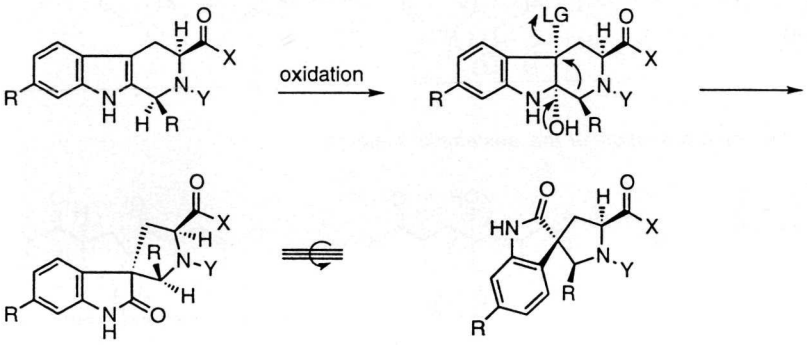


Scheme 2.2: *Hypothetical biosynthetic pathways to (reverse) prenylated indoles.*

from *Aspergillus fumigatus*[55] that are cytotoxic against certain leukemia cell lines contain L- and D-alanine building blocks, respectively.

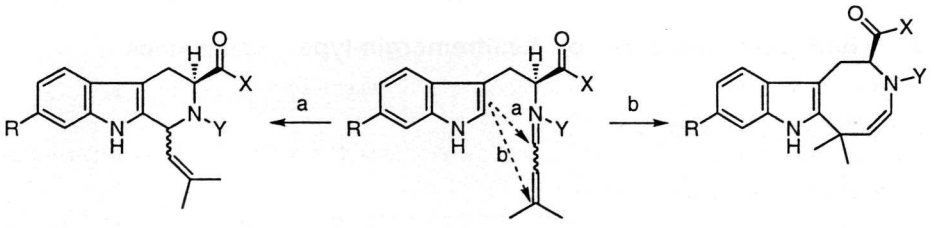
In the course of a screening for inhibitors of the mammalian cell cycle, spirotryprostatins A (**21**) and B (**22**) were isolated from *Aspergillus fumigatus*. These compounds were of a novel construct with a unique spiro ring system.[56],[57] They are formally derivable from tetrahydro-β-carbolines *via* oxidation of the indole 2-position followed by rearrangement (Scheme 2.3), and spirotryprostatin A (**21**) is actually an oxidized form of

fumitremorgin C (**3**). Elegant total syntheses towards compounds of this spiro-oxindole class have been described.[58],[59]



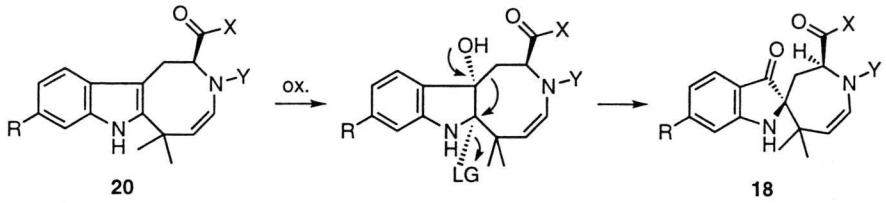
Scheme 2.3: *Formation of the spiro-oxindole framework of the spirotryprostatins.*

In the light of this route, which might have biosynthetic relevance, the frameworks of other classes can be explained as well (Scheme 2.4). A suggested biosynthetic pathway of the spirotryprostatins follows that of the fumitremorgins prior to the oxidation and rearrangement steps, i.e. a normal Pictet-Spengler-like sequence (pathway a) involving a common α,β-unsaturated imine intermediate. The natural occurrence of verruculogen TR-2 (**7**), possessing a hydrated isoprenyl (hydroxyisobutyl) sidechain, however suggests the possibility of Michael addition of water to the α,β-unsaturated imine prior to Pictet-Spengler type cyclization. Alternatively, an unprecedented conjugate cyclization of the α,β-unsaturated imine (pathway b) could be a key step in the formation of natural product (**20**).[60] Oxidation of (**20**), similar to the spirotryprostatin case but with the positions of the oxygen and the leaving group interchanged, and subsequent rearrangement then could lead to the austamide class of alkaloids (Scheme 2.5).

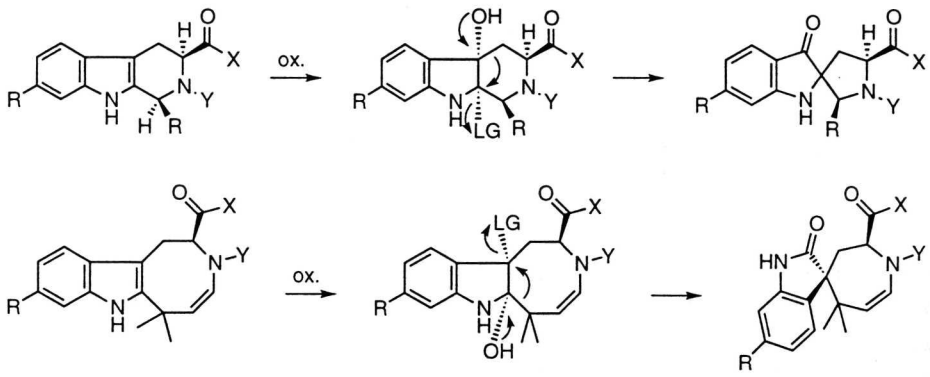


Scheme 2.4: *Mode of cyclization determines the compound class.*

We thought that combination of the two biosynthetic modes described above could hypothetically lead to two other classes of alkaloids (Scheme 2.6). Nonetheless, to the best of our knowledge, both of them have not been reported in literature to date.



Scheme 2.5: *Assumed formation of the austamide skeleton.*



Scheme 2.6: *Fictive alkaloids that could be formed via identical mechanisms.*

The examples shown above, which by no means form a complete overview, are all cyclic core structures that can be viewed as natural scaffolds for displaying amino acid sidechains in spatially different positions. Not only the natural products themselves, but likewise analogs based on these templates might show interesting biological activity and thus are prevalent targets for total synthesis.

2.2 Biological activities of fumitremorgin-type compounds

2.2.1 *Tremorgenic activity*

2.2.1.1 *Physiological effects*

The outbreak of diseases among farm animals that had consumed molded food was the initiation of the research that led to the discovery of the interesting class of fumitremorgin-type indole alkaloids. Fungus cultures present on foodstuffs that had been consumed prior to manifestation of tremors were examined in order to reveal the secondary metabolites responsible for the neurotoxic properties. Mycotoxin-induced intoxication leads

to typical symptoms like diminished activity and immobility, followed by hyperexcitability, convulsions, muscle tremor, ataxia and tetanic seizures. In non-lethal cases, these symptoms are reversible if the affected animal is removed from the toxic feed source. Since the tremorgenic mycotoxins are relatively lipophilic molecules, they may cross the blood-brain barrier and enter the central nervous system (CNS) to trigger a reaction.

The first compounds of the previously unknown class of indolyl diketopiperazine alkaloids that exhibited trembling-inducing activities were fumitremorgins A (**1**) and B (**2**). Intraperitoneal (IP) injection of 1 mg of these compounds in mice caused, after five minutes, sustained trembling with intermittent convulsion that lasted for several hours. Fumitremorgin B (**2**) was found to cause more severe convulsion than fumitremorgin A (**1**). Though no lethality was observed with a dose of 1 mg, administration of 5 mg per mouse caused 70% of the observed animals to die within 96 hours.[7] The structurally simpler fumitremorgin C (**3**) has been reported to induce tremors in cockerel after oral administration of 25 mg/kg.[61] Verruculogen TR-1 (**5**), the most potent of all tremorgenic mycotoxins known, exhibited similar properties, and tremor persistence showed to be dose-dependent. Other clinical signs observed were hypersensitivity to sound, tetanic spasms and ataxia, and all signs were exaggerated by enforced movement or fright. The threshold response to the toxin after IP administration was similar for mice and cockerels; the ED_{50} was determined to be 0.39 mg/kg IP in mice and 0.33 mg/kg IP in cockerels.[62] Oral administration required 40 or 75 fold dosages, respectively, to sort the same effect. The compound showed an LD_{50} of 2.4 mg/kg in mice and 15.2 mg/kg in cockerels after IP injection, whereas oral administration gave LD_{50} values of 126.7 and 365.5 mg/kg, respectively. Mice receiving lethal doses of verruculogen TR-1 (**5**) usually died during a tetanic convulsion within 45 minutes after treatment. Gross examination at autopsy revealed no apparent mode of action. Animals receiving sub-lethal doses appeared to be fully recovered between 24 to 48 hours after treatment.[8] Verruculogen TR-2 (**7**), the hydrogenation product of verruculogen TR-1 (**5**),[9] produced perceptible tremors in cockerels dosed orally *via* crop intubation at dosage levels down to 12.5 mg/kg,[12] which makes it at least equipotent to its parental structure (**5**). This suggests that the substitution pattern on the south side of the molecule is not of vital importance to the tremorgenic activity.

2.2.1.2 *Mechanism of action*

Efforts have been made to determine the mode of action of these fungal tremorgens. It is perceived to involve inhibition of the presynaptic release of γ-aminobutyric acid (GABA) at a certain neuromuscular junction in the CNS.[63] Though fumitremorgin-type compounds have proven to be valuable tools in CNS function research,[64],[65],[66],[67] particular molecular features accounting for tremorgenic activity have not been completely identified. However, there are indications that the conformation and the configuration of the diketopiperazine moiety affects the activity.[65] In this light, the proline residue on the east side of the molecule may play an important role in maintaining the diketopiperazine in the right conformation.

Intravenous injection of fumitremorgin A (**1**) not only causes tremor and generalized tonic-clonic convulsion,[64],[68] also the autonomic nervous system is affected.[69] These effects may all be the result of abnormal excitation of the CNS. However, an electroencephalographic (EEG) seizure pattern such as present in a grand mal type of epilepsy is not observed. Since the convulsions also manifest in decorticated or decerebrated rabbits, it has been suggested that the main site of action may be in some restricted areas in the lower part of the CNS, such as the spinal cord or the brain stem.[69] In 1989, a CNS study in rabbits focussing on the effects of fumitremorgin A (**1**) on the spinal cord was reported.[70] It was concluded that the remarkable motor effect has its origin in the supraspinal CNS, some regions in the brain stem from the mesencephalon to the medulla, rather than in a direct facilitatory action on spinal motoneurons. This view is supported by the fact that drugs known to have potent inhibitory activity on the brain stem, such as pentobarbital, chlorpromazine and diazepam, could antagonize the tremorgenic effect of fumitremorgin A (**1**). Additional support can be found in the increase in verruculogen TR-1 (**5**) induced spontaneous release of aspartate and glutamate from rat cerebrocortical synaptosomes but not from spinal cord/medullary synaptosomes.[63] Assuming that these excitatory amino acids play a key role in the motor effects, these results suggest the main site of action to be in a certain part of the higher CNS rather than in the spinal cord. The effect of fumitremorgin A (**1**) on the brain stem was described in 1990.[71] A facilitatory effect on some neurons in the midbrain reticular formation was observed at intravenous (IV) doses of 20-50 μg/kg; abnormal activation of these neurons, brought about by IV injection of 200 μg/kg fumitremorgin A (**1**), results in convulsive burst discharges in peripheral motor nerves. A role of the medullary reticular formation could not be excluded. There is no precise explanation about the mechanism by which the excitability of the reticular formation is increased. Since diazepam and pentobarbital –having binding sites known to be closely related to the GABA receptor– are able to inhibit fumitremorgin A (**1**) induced convulsions, a central GABAergic neuronal mechanism may be involved. Support for this hypothesis comes from the report that verruculogen TR-1 (**5**) decreases the GABA level in the CNS, which may be an important pathophysiological process in the convulsions.[72] It has also been postulated that these agents could act as GABA mimics, since a rigid GABA-like conformation can be recognized within the structure of the fungal tremorgens.[73] On the other hand, the excitatory amino acids aspartate and glutamate are also suggested to play a role,[63],[74] and the real involvement of various neurotransmitters in convulsions induced by fumitremorgin-type compounds thus remains to be investigated further. On a peripheral level, the indole alkaloid tremorgens also appear to have an effect on neurotransmitter release mechanisms. For instance, verruculogen TR-1 (**5**) causes an increase in electrical field stimulated contractile responses in guinea pig ileum preparations. This effect was attributed to an enhanced acetylcholine release from presynaptic nerve terminals.[75]

All together, the fumitremorgin-type alkaloids seem to have multiple effects on receptors and on neurotransmitter release mechanisms, both central and peripheral. This raised the question whether effects on ion channels might, in part, be involved in the effect of these tremorgenic mycotoxins on neurotransmitter release. A 1994 report[76] describes the

investigation on the role in this process of so-called maxi-K channels, high-conductance Ca^{2+}-activated K^+ channels that in some neuronal preparations have been shown to regulate neurotransmitter release by controlling the duration of action potentials. Tremorgenic mycotoxins were shown to modulate ligand binding to receptors that are part of the maxi-K channel complex from aortic smooth muscle on a previously unknown interaction site, by either positive or negative allosteric mechanisms. Nanomolar concentrations of verruculogen TR-1 (**5**) markedly enhanced ligand binding in a concentration-dependent fashion. Studies with solubilized and partially purified receptor preparations suggest that this phenomenon is not due to physical perturbation of the membrane environment in which the receptor is located. Furthermore, the specificity of this interaction is illustrated by the fact that no effect was observed on other, voltage-dependent potassium channels located in the brain up to 100 μM concentrations. Both positively and negatively allosteric modulators, having opposite effects on ligand binding, are inhibitors of maxi-K channels, although the latter class seems to consist of the most potent blockers. Complete block occurs at concentrations around 10 nM. The fact that both tremorgenic and non-tremorgenic alkaloids have shown to be potent maxi-K channel blockers however makes it unlikely that block of maxi-K channels is related to the tremorgenic effect, unless maxi-K channels in neural tissue exist with a pharmacological profile distinct from those in smooth muscle tissue. Notwithstanding, some of the pharmacological properties of these compounds could be related to their ability of efficiently blocking maxi-K channels. Broadening of action potentials at nerve terminals by preventing repolarization, which leads to an increase in neurotransmitter release, may be an example of this.

2.2.2 *Inhibition of the mammalian cell cycle*

2.2.2.1 *Structure – activity relationships*

In 1995, the first report appeared in which a novel activity of indolyl diketopiperazine alkaloids was mentioned: tryprostatin A (**9**) and B (**10**) were isolated in the course of a screen for new inhibitors of the mammalian cell cycle.[27] In this assay, the temperature-sensitive tsFT210 mouse mammary carcinoma cell line was used, which grows at 32°C; the cell cycle processes throughout G1, S, G2 and M phases. Incubation at 39°C causes the cell to be arrested at the late G2 phase, making synchronization possible.[77] Tryprostatin A (**9**) showed complete inhibition of cell cycle progression at the G2/M phase of tsFT210 mouse cells at a concentration of 50 μg/ml, whereas tryprostatin B (**10**) was more potent in this respect with a value of 12.5 μg/ml. In a following study,[28] involving a series of seven fumitremorgin-type secondary metabolites, the inhibitory effects on the cell cycle were studied in more detail, resulting in the data depicted in Table 2.1.

Table 2.1: *Inhibitory effects of indolyl diketopiperazines on cell cycle progression.*[a]

Compounds	9	10	4	3	8	2	5
MIC[b] [μM]	16.4	4.4	0.45	4.1	60.8	26.1	12.2
IC_{50}[c] [μM]	78.7	18.8	1.78	14.0	>243	>209	>196

[a] To determine the DNA content and calculate the distribution within the cell cycle, cells were analyzed by flow cytometry.
[b] MIC = Minimal Inhibitory Concentration.
[c] IC_{50} = 50% inhibitory concentration, as determined from dose-response curve.

Comparing the structures, it becomes clear that substitution of the C-12 and C-13 hydrogens for a hydroxyl group (**2**, **5**, **8** vs. **3**, **4**, **9**, **10**) significantly reduces inhibition of cell cycle progression in the M phase. Introduction of a methoxy group at C-18 (**3** vs. **4**, **9** vs. **10**) also markedly decreases the inhibitory activity, whereas closure of the central ring (**3** vs. **9**, **4** vs. **10**) is favorable. Morphological studies showed that cells treated with the four most potent compounds (**3**, **4**, **9**, **10**) were mainly arrested in the M-phase. Kinase assays in order to investigate the cellular target excluded direct inhibition of cdc2 kinase, which is essential to action of the maturation promoting factor (MPF) for escaping the G2/M boundary of the cell cycle. Furthermore, protein kinase A (PKA), protein kinase C (PKC) and the epidermal growth factor (EGF) receptor were not inhibited.[28] As demonstrated in a more detailed study,[29] casein kinase I (CK-I) is not inhibited by these compounds either. These results, coupled with the morphological characteristics of the treated cell nuclei, makes the mitotic apparatus a likely target for compounds (**3**), (**4**), (**9**) and (**10**). Antimicrobial activity of the compounds was studied as well, but turned out to be negligible.[28]

In 1996, a separation procedure of secondary metabolites from the fermentation broth of *Aspergillus fumigatus* guided by cell cycle inhibitory activity led to the discovery of spirotryprostatins A(**21**) and B (**22**). The bioassay using tsFT210 cells showed spirotryprostatin B (**22**) (IC_{50} 14.0 μM) to be more potent than spirotryprostatin A (**21**) (IC_{50} 197.5 μM). At 34.4 μM, spirotryprostatin B (**22**) completely inhibited cell cycle progression, whereas spirotryprostatin A (**21**) could inhibit the most portion of the cells in the G2/M phase at a concentration of 253.2 μM.[57] Again, the methoxy substituent on the aromatic ring seems to influence cell cycle inhibition negatively, although related examples (*vide supra*) underestimate the difference in activity between **21** and **22**. For instance, the presence or absence of the double bond that further distinguishes spirotryprostatins A(**21**) and B (**22**) may be of influence as well. A 1999 report on synthesis of spirotryprostatin analogs and their evaluation as cell cycle inhibitors[59] revealed interesting structure activity relationships that go beyond the presence or absence of a methoxy group on the aromatic ring. Though dependent on the type of human breast cancer cells used, analogs of less complex structure show significantly higher activity than their parental natural compound spirotryprostatin A (**21**). Removal of the methoxy group from (**21**) only resulted in minor improvement in activity. Substitution of the isoprenyl sidechain for the corresponding external olefin also gave slightly lower IC_{50} values, whereas demethoxy-spirotryprostatin A with the isoprenyl substituent replaced by a benzyl group shows a striking increase in activity. Most remarkably,

a synthetic precursor of the latter compound –lacking the proline-containing diketopiperazine, and having the remaining amino acid nitrogen Boc-protected and the carboxylic acid protected as a methyl ester– is an equipotent cell cycle inhibitor. Thus, at least for this specific class of spirooxindole compounds, both the nature of the sidechain and the diketopiperazine moiety seem to be of little relevance for cell cycle inhibitory activity.

In 1997, the cyclotryprostatins A (**11**), B (**12**), C (**13**) and D (**14**) joined the series of mammalian cell cycle inhibitors from the indolyl diketopiperazine class. Their data from the biological assay on the tsFT210 cells are portrayed in Table 2.2.[30]

Table 2.2: *Inhibition of mammalian cell cycle progression by cyclotryprostatins A-D.*

Compounds	**11**	**12**	**13**	**14**
IC_{50} [μM]	5.6	19.5	23.4	25.3

Comparing cyclotryprostatin C (**13**) with dihydroxyfumitremorgin C (**8**), the negative effect of the C-18 methoxy substituent again emerges. The highly potent cyclotryprostatin A (**11**) is the C-12 epimer of the very weakly active dihydroxyfumitremorgin C (**8**). It is even a stronger inhibitor than cyclotryprostatin C (**13**), differing in stereochemistry at C-12 and the C-18 methoxy substituent. These observations suggest a prominent role of the stereochemistry at C-12, which is important for the conformation of the central and the diketopiperazine rings.

2.2.2.2 *Mechanisms of action*

The mechanism by which tryprostatin A (**9**) inhibits mammalian cell cycle progression was revealed in 1998.[78] Tryprostatin A (**9**) inhibited cell cycle progression of asynchronously cultured rat normal fibroblast 3Y1 cells in the M-phase in a dose- and time-dependent manner. Tryprostatin B (**10**), lacking the methoxy substituent of (**9**), in contrast, showed cell cycle non-specific inhibition on cell growth, even though the threshold concentration for cell growth inhibition is lower than that for (**9**). A more extensive study on cell cycle progression *in situ* and microtubule assembly *in vitro* including the tryprostatins (**9**, **10**) and the cyclotryprostatins (**11**, **12**, **13**, **14**)[79] confirmed that the presence of the methoxy group reduces general cytotoxicity. The demethoxy compounds (**10**, **13**, **14**) were all highly cytotoxic at 250 μM. To illustrate the difference, the IC_{50} for the methoxy bearing tryprostatin A (9) was ~400 μM, whereas its demethoxy analog tryprostatin B (**10**) showed a 100-fold lower IC_{50} of ~4 μM. On the other hand, the DNA distribution of the studied cells indicated that the methoxy substituent on the aromatic part enhances the M-phase specificity.

Since it is known that many M-phase inhibitors affect the function of the spindle apparatus *via* microtubule disassembly, the influence of tryprostatin A (**9**) on targets known to be involved in microtubule processing by the cell was investigated. Microtubules are dynamic structures, continuously undergoing assembly and disassembly. Their function is to determine cell shape and assist a variety of cell movements including the separation of chromosomes during mitosis. Microtubules are composed of a single type of globular protein, called tubulin.

This is a dimer of two closely related polypeptides, α-tubulin and β-tubulin. These tubulin dimers polymerize to form microtubules, rigid hollow rods approximately 25 nm in diameter. Compounds like colchicine[80] and the *vinca* alkaloid vinblastine[81] bind tubulin, thereby inhibiting microtubule polymerization, which in turn blocks mitosis. Taxol[82] and the epothilones,[83] in contrast, stabilize microtubules, thereby disturbing the dynamic structures and thus blocking cell division. The dynamic behavior of microtubules is regulated by a series of microtubule-associated proteins (MAPs), including MAP1, MAP2 and tau (τ). These proteins bind to microtubules and inhibit the dissociation of tubulin subunits. In addition to this microtubule stabilizing effect, they can mediate their association with other elements of the cytoskeleton.

To follow the effect of tryprostatin A (**9**), the cytoplasmic microtubule network was observed *in situ* by indirect fluorescence microscopy.[78] The compound showed reversible, dose-dependent depolymerization of the microtubules, containing both cytoplasmic network and spindle apparatus. At 20 μM concentration, the network was completely disrupted in 6 h., whereas treatment with 50 μM had the same effect within 3 h. Withdrawal of the inhibitor resulted in microtubule reassembly within 3 h. These results suggest M-phase inhibitory effects due to interference with spindle apparatus function via microtubule disassembly. Microtubule assembly in vitro from a 2 mg/ml concentration of microtubule proteins was inhibited dose-dependently (40% inhibition at 250 μM). Binding competition experiments with [^{3}H]colchicine and [^{3}H]vinblastine showed no effect of tryprostatin A (9) on binding of the two labeled compounds on their distinct β-tubulin binding sites, indicating interaction on a different site of tubulin. The microtubule-associated proteins MAP2 and tau, that induce tubulin assembly *in vitro* through binding to the C-terminal domain of tubulin, were blocked in the presence of 250 μM tryprostatin A (**9**). Apparently, tryprostatin A inhibits microtubule assembly by interfering with interactions between microtubule-associated proteins and the C-terminal domain of tubulin. Results from the more extensive study[79] showed that microtubule assembly *in vitro* was reduced in the presence of 250 μM of (**9**): 58% of control; (**10**): 33%; (**11**): 66%; (**12**): 66%; or (**13**): 51%. Interesting is that cyclotryprostatin D (**14**), which differs from cyclotryprostatin C (**13**) only at the C-13 position, on the contrary promoted microtubule assembly up to 137% of control. In the light of these results, it seems likely that tryprostatin derivatives act as antagonists (or in the case of (**14**) as agonists) of MAP or tau proteins, making them a new type of lead compounds for antimitotic and antitumor drugs.

In 2000, the synthesis and biological evaluation of a series of prenylated derivatives of *cyclo*-L-Trp-L-Pro (the diketopiperazine of L-tryptophan and L-proline, also known as brevianamide F) as simple analogs of tryprostatin B (**10**) was published.[84] The parent compound *cyclo*-L-Trp-L-Pro was inactive in cell cycle inhibition and microtubule assembly assays at 250 μM, whereas an isoprenyl substituent on either the indole nitrogen or the diketopiperazine nitrogen only results in slight inhibition of cell proliferation at concentrations above 500 μM; neither of the compounds however inhibited *in vitro* microtubule assembly. In contrast, isoprenyl-substitution of both available nitrogens resulted in high cytotoxicity down to 50 μM concentrations, and only 4.6% *in vitro* microtubule assembly at 50 μM.

Another report[85] described the synthesis and evaluation of a series of analogs, including pentacyclic demethoxy-fumitremorgin C skeletons with different substituents on the original isoprenyl position. The stereochemistry of these substituents was found to be of significant importance, since for alkyl substituents (both the natural isoprenyl sidechain and its dihydro form) the natural *cis*-configuration is active, whereas the *trans* epimers are inactive. In the case of aryl sidechains, the situation becomes more complicated, since with increasing substitution of the aromatic ring a preference for *trans*-configuration seems to arise. The precise underlying details remain to be investigated, but it is envisaged that the shift in stereochemical preference might reflect a change in the mechanism of action due to closer resemblance to the synthetic podophyllotoxin-analog azatoxin.

2.2.3 *Reversal of multidrug resistance*

2.2.3.1 *ABC transporters*

Molecular transport across cellular membranes is of vital importance for all organisms. Many specialized compounds in prokaryotes, eukaryotes and archae-bacteria are actively –at the expense of ATP– transported by so-called ATP-binding cassette transporters (ABC transporters), the protein products of the large superfamily of ABC genes.[86] Such systems are known to be involved in a wide range of biological processes, including for example ion transport and pathways to provide essential nutrients such as sugars and amino acids to cells. Furthermore, they can function as a pump mechanism to protect vital organs such as the brain from a wide range of noxious compounds. Moreover, they are involved in processes controlling uptake (for example in the intestine) and excretion (for instance in kidney and liver) of potentially toxic foreign compounds. Mutations in ABC transporters designed as carriers for specific endogenous compounds have been shown to underlie several human inherited diseases.[87]

ABC transporters are one of the few superfamilies abundant in all three kingdoms of life,[88] and are characterized by hydrophilic 200-250 amino acids nucleotide binding domains (NBDs) that are extensively conserved throughout evolution. These harbor two short peptide motifs involved in ATP binding (Walker A and Walker B), with in-between the unique 'ABC signature' that can be used to identify members of the ABC superfamily.[89] A functional ABC transporter normally consists of two of these ATP binding cassettes (ABCs) and two sets of transmembrane domains (TMDs). They are assumed to function co-operatively by an alternating sites mechanism,[90] since inactivation of one catalytic site completely abolishes all activity.[91] Two types of transporters can be distinguished: full transporters which have all four domains within a single polypeptide chain (often a single, tandemly duplicated molecule), or half transporters with a single transmembrane and a single ATP binding segment. The latter are assumed to form homo- or heterodimers in order to obtain a functional protein. As a general rule, full transporters are mostly located in the plasma membrane of the host cell, while half transporters –in homo- or heterodimeric form– are targeted to the

membranes of subcellular organelles, like the endoplasmic reticulum, peroxisome or mitochondria.

Molecules to be transported recognize their own transporter (probably at the much less conserved transmembrane domain), bind and switch on the ATPase, which in turn activates or opens the transport pathway, either inwards or outwards. The ABC domains are molecularly coupled to the transmembrane domains to ensure the transmission of the conformational changes caused by substrate binding and by the hydrolysis of ATP to activate transport. These pathways may either involve a classical channel, a "gateway" mechanism through a proteinacious chamber spanning the bilayer, or conceivably *via* a pathway at the protein-lipid interface of the outside of the membrane domain.[92]

The extraordinary range of compounds that can be transported by different members of the family of ABC systems makes it a challenge to understand the basis of selectivity from one system to another. However, despite numbers of specific ABC transporters known, several systems have been identified which in contrast are remarkably a-specific. The most extensively studied are P-glycoprotein (P-gp1 or MDR1, generally described as P-gp) and the multidrug resistance associated protein (MRP1, often described as MRP).[93],[94],[95] It is generally accepted that these so-called multidrug transporters form an important part of an organism's natural defense system by preventing toxic substances from entering certain tissues or accumulating in particular organs.

2.2.3.2 *Multidrug resistance*

Among the ABC transporters, especially the multidrug transporters have become a topic of active research, since several clinically important issues can be associated with them. For instance, among the members of the ATP-binding cassette transport protein superfamily (ABC transporters),[86] there are several plasma membrane proteins associated with multidrug resistance (MDR) of cancer cells when overexpressed. Multidrug resistance describes the phenomenon of simultaneous cross-resistance to a range of structurally and/or functionally unrelated drugs when a cell shows resistance to a single cytotoxic drug.[96] Drug-naive tumors may manifest at the outset of chemotherapy (innate drug resistance),[97] or may develop later in patients who achieve an initial remission (acquired drug resistance). The latter case can be effected by the use of a single drug (a stress condition for the cell), inducing expression of a certain multidrug transporter. The genes for the multidrug transporters are present in each cell, but are normally brought to significant continuous expression only at the barriers of tissues that need to be protected. It is likely that drug-resistance mechanisms, mechanisms routinely used by certain normal cells for defense against xenobiotics, will be enlisted by tumor cells for their own protection. An increase in resistance often occurs after prolonged exposure to drugs, and may involve gene amplification and/or genetic changes either in the structural gene or in regulatory components of the MDR systems.[98] The cells with elevated MDR transporter expression will be less sensitive to treatment due to reduced drug accumulation, and will soon form the major population. Elevated levels of multidrug transporters, that often recognize a broad spectrum of drugs, result in efficient excretion of the

therapeutic agents. Clinical resistance of cells to chemotherapeutics, a major problem in the treatment of cancer, may in part be due to enhanced activity of these broad specificity drug efflux pumps, like P-glycoprotein (P-gp) and the multidrug resistance associated protein (MRP1).[99]

2.2.3.3 *The multidrug-transporter P-glycoprotein*

As stated before, one of the physiological roles of these broad specificity efflux pumps is generally thought to be elimination of toxic compounds, for instance by preventing them from crossing tissue barriers or building up high intracellular concentrations. This is based on the localization of P-glycoproteins in the plasma membrane, on the apical (or luminal) surface of polarized epithelial cells. These include the brush border membrane of intestinal cells, the biliary canalicular membrane of hepatocytes, and the luminal membrane in proximal tubules of the kidney.[100] Their presence at the pharmacological barriers of the body, e.g. at the blood-brain barrier,[101] the testicular and placental endothelium, and at the choroid plexus[102], supports this view. Besides naturally occurring carcinogens such as benzo(*a*)pyrene[103] and physiological substances such as certain hormonal steroids,[104] the spectrum of substrates transported by P-gp includes several drugs widely used in cancer chemotherapy. Compounds known to be affected by P-gp include doxorubicin, mitoxantrone, vincristine, vinblastine, paclitaxel and topotecan, but not drugs such as bleomycin, methotrexate, cisplatinum and several alkylating agents.[105] Lipophilicity seems to be an important requirement for a compound to be expelled by P-gp. The precise efflux mechanism is still an area of active research. There is mounting evidence for a model of action in which the transmembrane segments directly interact with a broad range of hydrophobic compounds present between the phospholipids of biological membranes, resulting in direct transport to the exterior of the cell with aid of the ATPase unit.[92] The second determinant of drug 'specificity' is then the subsequent interaction with drug binding sites at the transporter protein. Kinetic analysis of drug dissociation revealed the presence of two non-identical, allosterically linked drug binding sites in P-gp.[106] There are indications that the transporter contains multiple non- or partially overlapping binding sites, each having different affinities for different (classes) of drugs.[105]

Elegant studies using homologous recombination inactivation in knock-out mice have provided insight into the function of the P-gp encoding gene families.[107] Knockout of the equivalent of the human *mdr1* gene in mice has demonstrated that this gene is involved in the blood-brain barrier and in drug transport in the intestinal tract.[108] Though the knockout mice were viable and fertile, with no obvious physiological abnormality, they were found to be hypersensitive to xenobiotic compounds. Likewise, the mouse analog of the human *mdr2/3* gene –closely related to MDR1 but probably with a minor role in multidrug resistance– was shown to play a role in phosphatidylcholine transport in the bile canaliculi of the liver,[109] demonstrating that different P-gp isoforms have different functions.

2.2.3.4 *The Multidrug Resistance associated Protein*

A significant part of the non-P-gp mediated MDR might be due to MRP1 overexpression. MRP1 is suggested to play a role in detoxification by excretion of endogenous and environmental toxicants. Apart from the taxanes and mitoxantrone, which are not transported by MRP1, the spectrum of resistance imparted by MRP1 overexpression is very similar to that of P-gp.[110] Drug substrates for MRP1 either are anionic glutathione conjugates, or hydrophobic compounds that are thought to be co-transported with glutathione.[111] Depletion of glutathione, for instance by applying buthionine sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthase involved in glutathione biosynthesis, sensitizes MRP1 overexpressing cells to MRP1 substrate drugs.[110] MRP1 is routed to the basolateral membrane in polarized kidney and epithelial cells. A substantial fraction is not in the plasma membrane but located in intracellular vesicles including the endoplasmatic reticulum and Golgi apparatus,[110],[112] thereby forming an extra barrier for drugs for reaching their cellular targets. As MRP1 is located at the basolateral side of epithelium cells, it tends to pump substrates into the body, rather than into bile, urine or gut for disposal, as P-gp (located at the apical side) does. MRP1 therefore especially manifests in organs that require a basolateral transporter for protection, such as bone marrow, testis, kidney, the choroid plexus and the oropharyngeal mucosa. Besides providing xenobiotic resistance, MRP1 –being the major high-affinity transporter of leukotriene C_4– seems to play a key role in inflammatory response.[113] Enforced expression of two other MRP family members, MRP2 (also called cMOAT)[114] and MRP3 (also known as MOAT-D)[115] has also shown to result in resistance of cells to multiple drugs. The clinical significance of high MRP1 levels found in some human tumors remains to be established.[116]

While P-gp appears to consist of similar C- and N-terminal halves each containing six transmembrane helices,[117] as is the case for most full transporters,[86] MRP1 has an additional lipophilic N-terminal segment of about 230 amino acids. Interestingly, some other members of the MRP family[118] lack this region, and full deletion of this region from MRP1 indicated that it is neither required for the transport function nor for its proper routing to the lateral plasma membrane compartment.[119] Another feature of the MRPs is a deletion in the N-terminal nucleotide binding domain of 13 amino acids in the ABC signature between the Walker A and B motifs, in comparison to P-gp.[116]

2.2.3.5 *Modulation of multidrug transporters*

Because of the narrow range of efficacy for cytotoxic drugs, a two-fold increase in cellular drug resistance could theoretically already be sufficient to develop clinical drug resistance. Inhibitors of these cell surface pumps thus are potential agents of clinical interest for resensitization of certain resistant cancer cells by maintaining lethal intracellular antineoplastic drug levels. Indeed, studies in certain types of cancers show that expression of known MDR transporters correlates with poor response to chemotherapy, and in some cases inclusion of reversing agents has improved clinical efficacy. On the other hand, in numerous

cases treatment in the presence of chemosensitizing agents fails, suggesting the existence of other MDR mechanisms.[120] The challenge is to determine which of the enormous amount of ABC transporter superfamily members present in the human genome are clinically relevant. For those, more potent and more specific inhibitors need to be developed, in order to obtain better clinical results than realized with "off-the-shelf" drugs that were designed for other indications but have serendipitously shown to act as P-gp directed MDR modulators.[121] These include calcium channel blockers, coronary vasodilators, indole alkaloids, quinolines, hormones, and immunosuppressants. Interestingly, for the first-generation P-gp inhibitor Verapamil (Figure 2.4) and other calcium channel blockers (an activity that does not correlate with anti-MDR activity), it was shown that both enantiomers are equally active chemosensitizers.[122]

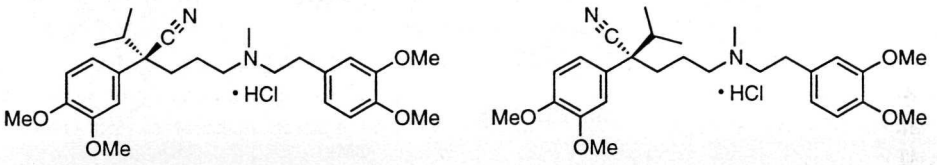


Figure 2.4: *The calcium channel blocker and first-generation P-gp inhibitor Verapamil.*

The doses of these first-generation modulators are generally limited by their toxic effects, which often occur below levels required to achieve effective transporter inhibition.[123] Although there are examples known in which combination of two chemosensitizers with non-overlapping toxicities, to achieve an overall anti-MDR effect greater than possible with individual agents, resulted in (supra)additive activity,[124] the need for compounds without intrinsic side effects remained. Unfortunately, the lack of detailed crystallographic structural information of membrane bound P-gp impedes a purely molecular approach to drug design for this target. Notwithstanding, several potent second generation agents (stereoisomers or analogs of first generation counterparts) and third generation agents (new compounds from rational or combinatorial approaches) are currently in development for modulating P-gp, including the non-immunosuppressive cyclosporine analog Valspodar (Amdray®) also known as PSC833 (**33**)[125] (Novartis), LY335979 (**34**)[126] (Eli Lilly), GF120918 (**35**)[127] (Glaxo-Wellcome), and XR9051 (**36**)[128] and XR9576 (**37**)[129] (Xenova) (Figure 2.5). Most of these compounds are lipophilic in nature, and share a broad structural similarity that includes a heterocyclic ring nucleus separated at a distance from an amino group that is cationic under physiological conditions. Kinetic studies using labeled compounds indicate that XR9576 (**37**) binds at a site which is distinct from the site of interaction of transport substrates,[130] i.e. the modulatory effect would be due to non-competitive inhibition by altering substrate binding or the transport process in an indirect or allosteric fashion. Non-competitive inhibition has also been shown for GF120918 (**35**).[131]

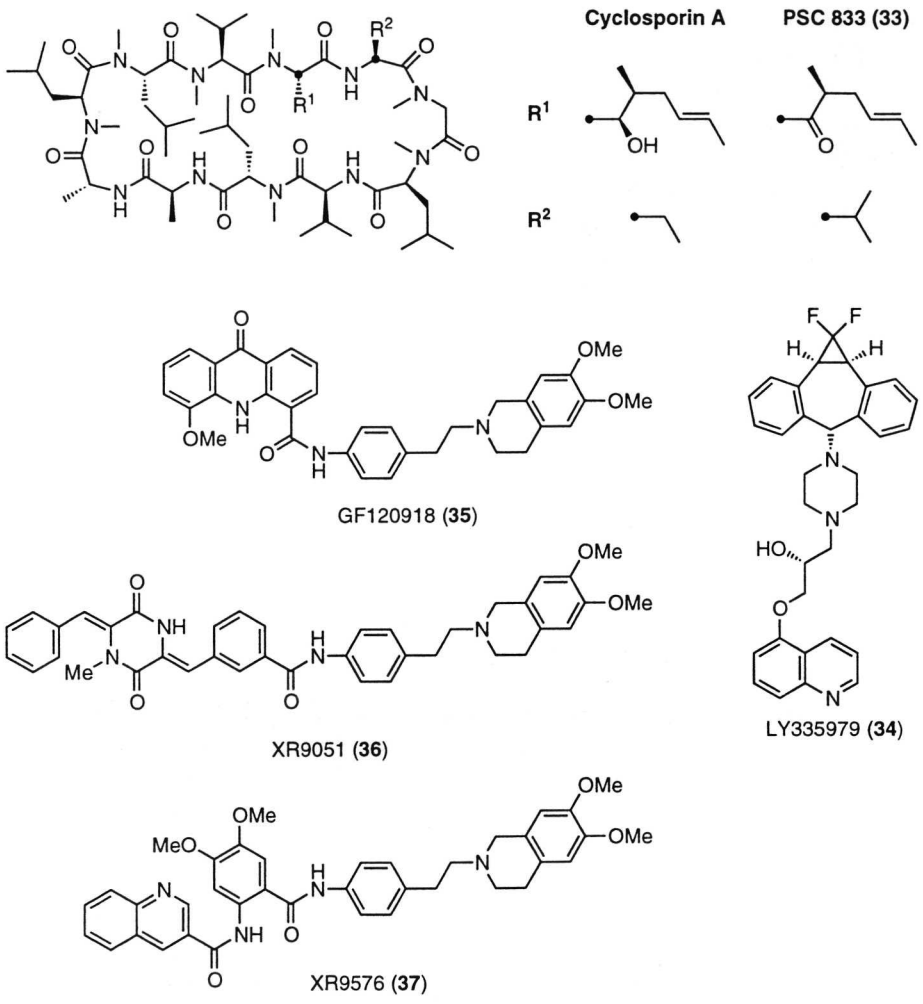


Figure 2.5: *Next generation inhibitors of P-glycoprotein.*

Besides their important role in (re)sensitization cells to chemotherapy, inhibitors of multidrug transporters could also prove useful for other applications. Inhibition of transporters naturally present at various tissue barriers, such as the blood-brain barrier, the intestine and the placenta, may open ways to alter the pharmacokinetics of certain drugs and thus improve the efficacy of conventional therapy. Coadministration of inhibitors of multidrug transporters could influence aspects such as bioavailability, clearance, or penetration to protected tissue compartments.[132] Ultimately, it may enhance the bioavailability of certain drugs[133] sufficiently to enable patient-invariable plasma levels *via* oral instead of intravenous dosing.[134] Suitable oral formulations of anticancer agents would be cost-efficient and more

convenient for the patient. The fact that knockout mice lacking P-gp show altered pharmacokinetics of drugs but appear to be physiologically normal[108c],[135] is in this regard reassuring, since it indicates that modulation of naturally present multidrug transporters to improve pharmacokinetics can be realized without seriously interfering in vital functions. This tolerance to MDR modulation though may in part be explained by other redundant mechanisms of transport. However, the natural function of these transporters in normal cells of course implicates a more serious potential source of side effects in chemotherapy. Upon modulation of MDR to sensitize innate drug-naive tumors or reverse acquired drug resistance, not only endogenous compounds or xenobiotics that are normally prevented from reaching certain tissues might then be able to cross the natural barriers and have adverse effects.[132] Also the anticancer drugs administered together could have different pharmacokinetic profiles and biodistribution properties[136] and thus may trigger new toxicities, which remains a concern for future clinical trials. Fortunately, with some third generation P-gp modulators that show increased specificity, such as LY335979 (**34**), little pharmacokinetic interactions have been observed.[126] This suggests that at least part of the alterations observed by older first and second generation modulators may have been due to blockade of transporters other than P-gp.

2.2.3.6 *The bacterial multidrug transporter LmrA*

Cancer chemotherapy has it roots in antimicrobial chemotherapy, and many concepts are applicable to both. Reversal of multidrug resistance may also become an important issue in antimicrobial treatment, since it becomes clear that certain types of resistance in bacteria, fungi, yeasts and parasites can also be attributed to enhanced activity of multidrug transporters.[92],[137],[138] For example, in 1996 resistance to cytotoxic compounds of *Lactococcus lactis*[139] (a bacterium used in dairy fermentations) was attributed to an ATP dependent half-transporter called LmrA.[140] This single multidrug transporter was shown to confer resistance to a record of eight classes of clinically relevant broad-spectrum antibiotics.[141] Given the exceptionally broad specificity of LmrA for antibiotics, the putative transfer of the *lmrA* gene to other bacteria in food and the digestive tract, this type of ABC multidrug transporters is a serious threat to the efficacy of valuable antibiotics.

Homology of LmrA to other prokaryotic and eukaryotic ABC transporters[142],[143] has been reported. It proved to be the first example of a prokaryotic structural and functional homologue of the human multidrug resistance P-glycoprotein MDR1, extruding a similar spectrum of amphiphilic cationic compounds. Furthermore, a range of compounds known to be P-gp modulators[144] also inhibited LmrA activity. Functional heterologous expression of LmrA in eukaryotic cells, leading to a drug-resistant phenotype virtually identical to P-gp mediated MDR, strongly implies that its ability to confer drug resistance is independent of any auxiliary proteins.[145] LmrA possesses high sequence conservation to each of the two halves of P-gp, particularly in the regions (e.g. the first cytoplasmic loop and TMD segments V and VI) that have been implicated as determinants of drug recognition and binding by human P-gp and mammalian homologues,[146] and functions as a homodimer.[147],[148] A somewhat unique feature of LmrA is its location in the plasma membrane, unlike most

functional (clusters of) half-transporters which are targeted to intracellular membranes.[88] Consistent with the suggestion that P-gp removes drugs from the membrane rather than from the cytoplasm, based on either the 'flippase' model (drug transport from the inner to the outer leaflet of the plasma membrane, from where they diffuse)[149] or the 'vacuum cleaner' hypothesis (substrate uptake from the plasma membrane),[150] evidence has been obtained that LmrA expels drugs from the inner leaflet of the lipid bilayer.[151] As is the case for P-gp,[106] kinetic analysis of drug dissociation presented evidence for two non-identical allosterically linked drug binding sites in the protein expressed in plasma membranes of insect cells.[145] Equilibrium binding experiments, photoaffinity labeling and drug transport assays[147] suggest that homodimeric LmrA mediates drug transport by an alternating two-site transport (two-cylinder engine) mechanism. A transport-competent high-affinity drug binding site is situated on the inner membrane surface, and a low-affinity drug–release site is located at the outer membrane surface. Driven by ATP hydrolysis, these sites are interconverted via a catalytic transition state intermediate in which the drug transport site is occluded.

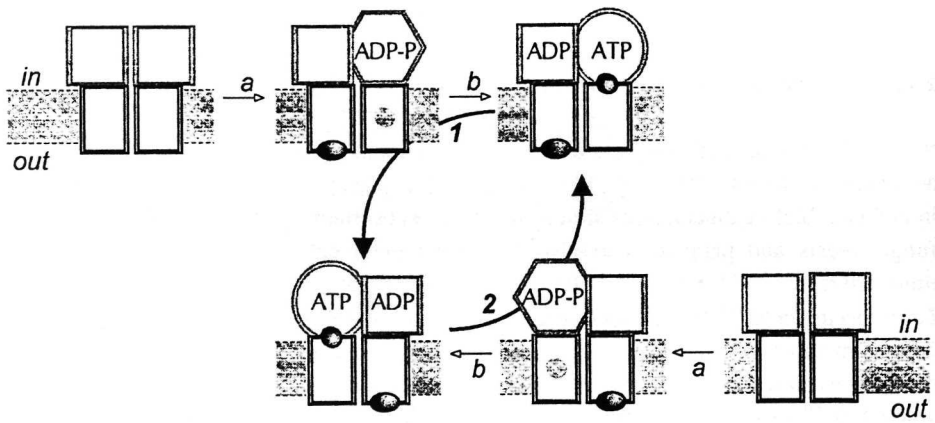


Figure 2.6: *Alternating two-site (two-cylinder engine) transport model.*[147]
Rectangles represent the transmembrane domains of LmrA. Circles, squares and hexagons represent different conformations of the nucleotide-binding domains. The ATP-bound (circle) state is associated with a high-affinity drug-binding site on the inside of the transporter. The ADP-bound (square) state is associated with a low-affinity drug-binding site on the outside of the transporter. The ADP-Pi (hexagonal) state is associated with an occluded drug-binding site, and represents the ADP/vanadate-trapped form of the ABC domain. In and out refer to the inside and outside of the phospholipid bilayer, respectively. The upper and lower horizontal branches (as indicated by arrows a and b) summarize the events that occur during equilibrium drug binding to the LmrA transporter. Arrow a: an initial drug-binding event with low affinity to one LmrA molecule in the dimer yields a binary drug–protein intermediate, which can be stabilized through the binding of Mg-ADP plus *o*-vanadate. Arrow b: a second drug-binding event with high affinity to the other LmrA molecule in the dimer yields a ternary drug–protein complex. In the presence of ATP, the transport cycle turns counterclockwise (as indicated by arrows 1 and 2). Arrow 1: ATP hydrolysis at the second ABC domain in the LmrA dimer is coupled to: (i) drug efflux at the second membrane domain through the movement of a liganded inside-facing high-affinity site to the outside of the membrane with the concomitant change to low affinity, via a catalytic transition intermediate in which the transport site at the second membrane domain is inaccessible; (ii) the reorientation of an empty outside-facing low-affinity site at the first membrane domain to an inside-facing high-affinity site; and (iii) ATP binding at the first ABC domain. Arrow 2: the first and second LmrA molecules in the dimer have reversed their relationship, and the next ATP hydrolysis step will occur at the first ABC domain. Thus, in a complete drug transport cycle, the LmrA dimer exposes four drug-binding sites in two pairs of two sites via an alternating two-site mechanism. It is important to be aware that although binding sites are presented in separate transmembrane domains in this scheme (for reasons of clarity), they may equally well be present at the interface between transmembrane domains.

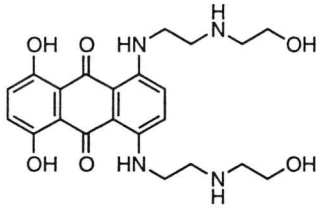
Interestingly, besides the P-gp homologue LmrA, *Lactococcus lactis* was also shown to possess an organic anion multidrug transporter with a substrate specificity remarkably similar to that of human MRP1.[152] Given the amount of horizontal gene transfer between intestinal flora and intestines, where a lot of multidrug transporter mRNA is expressed, it is not unlikely that other human (multidrug)transporters have bacterial homologues of potential clinical relevance as well.

2.2.3.7 *Breast cancer resistance protein, a new multidrug transporter*

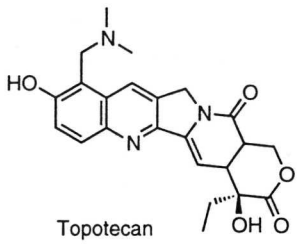
Recently, a new ABC transporter has been identified: the breast cancer resistance protein (BCRP),[153] also known as placenta-specific ABC transporter (ABCP)[154] or mitoxantrone-resistance gene (MXR)[155] product. The *BCRP* gene shows the highest homology to the *Drosophila* white (*w*) gene, which decodes a protein involved in transporting guanine and tryptophan[156] (31% identity with BCRP) and its human 638 amino acid homologue white/ABC8 (30% identity with BCRP). The *White* subfamily of ABC proteins[86] it belongs to also includes several fungal MDR genes; however, the TMD is rather distinct from its paralogs. BCRP/ABCP/MXR (from now on termed BCRP) is a 655 amino acid half-transporter located in the plasma membrane.[157] This is somewhat exceptional, since most half-transporters known are of eukaryotic origin and reside in the membranes of intracellular organelles. Furthermore, the configuration of the protein is different from the duplicated P-gp or MRP proteins; the latter share a NH_2-([TMD]-[NBD])$_2$-CO_2H topology, whereas in BCRP and other White subfamily members have the domains organized in a reversed order: NH_2-[NBD]-[TMD]-CO_2H. In normal tissues, BCRP expression is quite distinct from that of P-gp and MRP1. Human BCRP is naturally abundant in the placenta and in certain areas of the midbrain (putamen), and is expressed at lower to undetectable levels in a range of other normal adult tissues, although the highest expressing organs are liver, small intestine and colon.[153],[154],[158] Murine Bcrp1, in contrast, is highly expressed in kidney and more moderately in placenta and other tissues.[159] Though it is feasible that this transporter can play a role in the placental barrier, there is no specific endogenous substrate known. Its presence in the intestine and apically directed transport of drugs suggest a function as xenobiotic transporter to protect the body from harmful compounds. In contrast to P-gp however, tissue distribution studies revealed that murine Bcrp1 apparently only plays a role in the maternal-fetal barrier and not in the blood-brain and blood-testis barrier.[159]

Cross-resistance to therapeutically important drugs including mitoxantrone (MX),[160] doxorubicin, daunorubicin and topotecan (TPT) (Figure 2.7) due to reduced drug accumulation that is not P-gp or MRP1 related, has been reported for a number of drug-selected cell lines.[161],[162],[163],[164],[165],[166] These cells, generally MX- or TPT-selected, remain sensitive to vinca alkaloids, paclitaxel, verapamil and cisplatin. In several cell lines, this MDR phenotype has been attributed to elevated *BCRP* expression.[153],[154],[155],[167],[168],[169],[170] Reversal agents for this transporter thus are not only interesting from an academic point of view, but also potential agents of clinical interest. Strategic application of BCRP inhibitors may lead to more effective oral chemotherapy with BCRP substrate drugs *via* a dual mechanism:

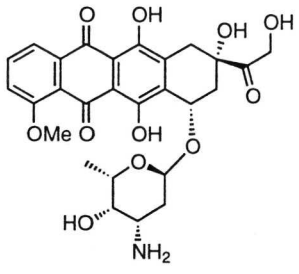
enhanced bioavailability by increased (re)uptake due to intestinal BCRP modulation,[171] and (re)sensitization of resistant tumors by inhibiting BCRP-mediated efflux from tumor cells.



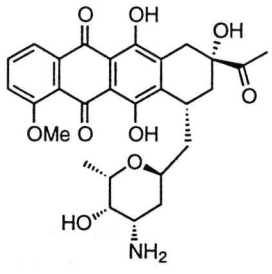
Mitoxantrone



Topotecan



Doxorubicin



Daunorubicin

Figure 2.7: *Antineoplastic drugs that are expelled by BCRP.*

Selected human tumor cell lines overexpressing *BCRP*, such as the resistant T8 subline derived from the human IGROV1 ovarian cancer cell line,[172] are of course important tools in understanding clinical, pharmacological and physiological roles of BCRP. A disadvantage is that the activity of the transporter might be masked or overshadowed by other resistance mechanisms such as P-gp or MRP1 activity. It was shown in 1999 that the mouse homologue of *BCRP*, *Bcrp1*, encodes a 657 amino acid protein Bcrp1 with 81% identity (86% similarity) to human BCRP which also is functionally comparable as a multidrug transporter.[173] Therefore, mouse models likely are appropriate and valuable for investigating the biochemistry and physiological functions of the BCRP/Bcrp1 protein, and its significance for drug pharmacokinetics and drug resistance in tumors. Especially mouse cell lines overexpressing Bcrp1 but lacking functional *Mdr1a* and *Mdr1b* (encoding mouse P-gp's) and/or *Mrp1* genes, such as the resistant T6400 subline derived from MEF3.8 embryo fibroblast cell line,[173] are highly valuable tools in *in vitro* experiments. For *in vivo* studies, the corresponding nullyzigous *Mdr1a/b*$^{-/-}$ [108c] and/or *Mrp1*$^{-/-}$ [113a] knockout mice can be of high importance. An extension of the research, comparing drug sensitivities of wild-type and knockout mice, was performed in order to investigate the role of the relatively low expression levels of MDR transporters found in most untreated tumors.[174] It revealed that P-gp and Mrp1, even at very low levels that can be difficult to detect, can significantly affect the innate

sensitivity of tumor cells to a wide range of clinically important substrate drugs. This implicates that use of resistance reversal agents to sensitize tumors may also be appropriate in cases where there is no *over*expression of multidrug transporters such as P-gp, MRP1 or BCRP.

Increased BCRP/Bcrp1-mediated efflux of mitoxantrone can be reversed by the Glaxo-Wellcome compound GF120918 (**35**)[131]. This compound was developed as a P-gp inhibitor[127] but, serendipitously, also inhibits BCRP. Clinically relevant substrate drugs of BCRP are often also P-gp substrates: P-gp confers a cross-resistance profile overlapping with BCRP for doxorubicin, mitoxantrone and other drugs.[158] Cells that overexpress the BCRP protein seem to be more resistant to mitoxantrone and topotecan than cells with P-gp mediated MDR.[175] Dual specificity inhibitors such as GF 120918 (**35**) may be clinically advantageous as a reversal agent/chemosensitizer for some drugs. In other cases it may prove a handicap, for example where P-gp in the blood-brain barrier[108a],[176],[177] protects against toxicity to the CNS. As an illustration of the latter, it has been shown that brain uptake and retention of P-gp substrates is significantly increased in *Mdr1a/b*$^{-/-}$ mice compared with the wild type, with no net change in blood pharmacokinetics.[178] Monospecific inhibitors of BCRP may thus prove useful also in the clinic. Furthermore, selective inhibitors of BCRP would be valuable laboratory tools for analysis of resistance mechanisms, for instance in revealing what transporter contributes most to MDR in certain types of tumors.

In 1998, by screening a library of extracts derived from a variety of microorganisms, fumitremorgin C (FTC, **3**) was identified as a specific and potent reversal agent for the non-P-gp, non-MRP related multidrug transporter now known as BCRP.[166] Using the mitoxantrone-resistant S1-M1-3.2 colon carcinoma subline, no toxicity of FTC (**3**) alone was observed in a dose range from 0.1 - 80 µM, but in combination with mitoxantrone 50% of the cells were killed in the presence of 0.35 µM FTC (**3**). Fumitremorgin A (**1**) and B (**2**), also present in the active extract, were found to be more toxic (20% cell death at 15 µM) and considerably less active (20- and 14-fold, respectively) in the reversal assay. A concentration of 5 µM FTC (**3**) markedly potentiated the toxicity of mitoxantrone, doxorubicin and of topotecan in the S1-M1-3.2 cell line, as well as in the MCF-7/mtxR and MCF-7/AdrVp breast cancer cell lines that show a MDR phenotype without P-gp or MRP overexpression. Tests on the P-gp overexpressing S1-B1-20 cell line and the MRP overexpressing HL-60/AR cell line showed no significant resensitization effect of FTC (**3**), indicating selectivity for BCRP.

Though FTC (**3**) has been used as a BCRP modulator in later studies using MX-selected 8226/MR human myeloma cell lines[167] and the BCRP-transfected MCF-7 breast cancer cells,[179] this mycotoxin has several disadvantages. First of all, isolation of this natural product from fungal sources only yields relatively limited amounts of compound.[28] Total syntheses of FTC (**3**) have appeared, [180],[181],[182] but are all relatively low-yielding multistep procedures in which introduction of the unsaturated sidechain is generally the highest obstacle. Even more important from a clinical point of view are the other known biological activities of the natural compound. Unfortunately, it is neurotoxic, being the structurally simplest bioactive member of the fumitremorgin/verruculogen/(cyclo)tryprostatin class of alkaloids that is known to induce tremors in vertebrate animals[183] (see § 2.2.1). At 25 mg/kg

oral, the mycotoxin causes tremors in cockerels.[61] Furthermore, FTC (3) causes inhibition of the mammalian cell cycle by means of arrest in the G_2/M phase[28] (see § 2.2.2). Less toxic and synthetically tractable analogs of FTC are thus of interest as specific BCRP inhibitors in the laboratory and as lead compounds for development of chemosensitizers/reversal agents for clinical use. Desirable properties of practical BCRP inhibitors include high potency in relation to toxicity, and specificity for the BCRP transporter. Pentacyclic analogs of demethoxyfumitremorgin C (4) bearing modified stereochemistry, side chains and ring sizes have been reported to inhibit enhanced BCRP activity in the S1-M1-3.2 cell line,[184] but the compounds in question tended to be less potent and more cytotoxic than native FTC (3).

2.3 Concluding Remarks

Indolyl diketopiperazine alkaloids of the fumitremorgin class unite two pharmacologically important moieties in a single core structure, that can be considered as one of nature's elegant solutions for displaying amino acid and isoprenyl-derived sidechains in spatially different positions. Several interesting biological activities have been found among members of this compound class, including interference with neurotransmitter release, cell cycle inhibition and reversal of multidrug resistance. Isolation of the alkaloids from natural sources yields limited amounts of compound, whereas their total syntheses have generally proven to be elaborative multistep procedures. Moreover, the complex interplay of multiple mechanisms of action these natural products have influence on illustrates the need for more selective, synthetically tractable analogs. Since the target structures are scaffold-sidechain like, development of a combinatorial approach would be the ideal method for obtaining and screening a substantial number of compounds.

2.4 References and Notes

1. Everett, G.M.; Blockus, L.E.; Shepperd, I.M. *Science* **1956**, *124*, 79.
2. Wilson, B.J.; Wilson, C.H. *Science* **1964**, *144*, 177-178.
3. Wilson, B.J.; Wilson, C.H.; Hayes, A.W. *Nature* **1968**, *220*, 77-78.
4. Ciegler, A. *Appl. Microbiol.* **1969**, *18*, 128-129.
5. Hou, C.T.; Ciegler, A.; Hesseltine, C.W. *Appl. Microbiol.* **1971**, *21*, 1101-1103.
6. Cole, R.J.; Kirksey, J.W.; Wells, J.M. *Can. J. Microbiol.* **1974**, *20*, 1159-1162.
7. Yamazaki, M.; Suzuki, S.; Miyaki, K. *Chem. Pharm. Bull.* **1971**, *19*, 1739-1740.
8. Cole, R.J.; Kirksey, J.W.; Moore, J.H.; Blankenship, B.R.; Diener, U.L.; Davis, N.D. *Appl. Microbiol.* **1972**, *24*, 248-256.
9. Cole, R.J.; Kirksey, J.W. *J. Agr. Food Chem.* **1973**, *21*, 927-929.
10. Yamazaki, M.; Sasago, K.; Miyaki, K. *J. Chem. Soc., Chem. Comm.* **1974**, 408-409.
11. Fayos, J.; Lokensgard, D.; Clardy, J.; Cole, R.J.; Kirksey, J.W. *J. Am. Chem. Soc.* **1974**, *96*, 6785-6787.
12. Cole, R.J.; Kirksey, J.W.; Cox, R.H.; Clardy, J. *J. Agr. Food Chem.* **1975**, *23*, 1015-1018.
13. Cole, R.J.; Kirksey, J.W.; Dorner, J.W.; Wilson, D.M.; Johnson Jr., J.C.; Johnson, A.N.; Bedell, D.N.; Springer, J.P.; Chexal, K.K.; Clardy, J.C.; Cox, R.H. *J. Agr. Food Chem.* **1977**, *25*, 826-830.
14. Willingale, J.; Perera, K.P.W.C.; Mantle, P.G. *Biochem. J.* **1983**, *214*, 991-993.
15. Yamazaki, M.; Fujimoto, H.; Akiyama, T.; Sankawa, U.; Iitaka, Y. *Tetrahedron Lett.* **1975**, 27-28.
16. In 1972, the structure determination of a compound isolated from *Penicillium lanosum*, named lanosulin, was reported [Dix, D.T.; Martin, J.; Moppett, C.E. *J. Chem. Soc., Chem. Comm.* **1972**, 1168-1169]. Though an alternative structure had been proposed, comparison with fumitremorgin B (2) showed that they were identical.[17]
17. Yamazaki, M.; Suzuki, K.; Fujimoto, H.; Akiyama, T.; Sankawa, U.; Iitaka, Y. *Chem. Pharm. Bull.* **1980**, *28*, 861-865.
18. Eickman, N.; Clardy, J.; Cole, R.J.; Kirksey, J.W. *Tetrahedron Lett.* **1975**, 1051-1054.
19. Yamazaki, M.; Fujimoto, H.; Kawasaki, T. *Tetrahedron Lett.* **1975**, 1241-1244.
20. Horak, R.M.; Vleggaar, R. *J. Chem. Soc., Chem. Commun.* **1987**, 1568-1570.
21. Cole, R.J. *J. Food Prot.* **1981**, *44*, 715-722.
22. Day, J.B.; Mantle, P.G. *Appl. Environm. Microbiol.* **1982**, *43*, 514-516.
23. Uramoto, M.; Tanabe, M.; Hirotsu, K.; Clardy, J. *Heterocycles* **1982**, *17*, 349-354.
24. O'Malley, G.J.; Cava, M.P. *Tetrahedron Lett.* **1987**, *28*, 1131-1134.
25. Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiotics* **1996**, *49*, 534-540.
26. Abaraham, W.-R.; Arfmann, H.-A. *Phytochem.* **1990**, *29*, 1025-1026.
27. Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Ubukata, M.; Takahashi, I.; Isono, K.; Osada, H. *J. Antibiotics* **1995**, *48*, 1382-1384.
28. Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Osada, H. *J. Antibiotics* **1996**, *49*, 527-533.
29. Osada, H.; Cui, C.-B.; Onose, R.; Hanaoka, F. *Bioorg. Med. Chem.* **1997**, *5*, 193-203.
30. Cui, C.B.; Kakeya, H.; Osada, H. *Tetrahedron* **1997**, *53*, 59-72.
31. Steyn, P.S. *Tetrahedron* **1973**, *29*, 107-120.
32. Steyn, P.S. *Tetrahedron Lett.* **1971**, 3331-3334.
33. Birch, A.J.; Wright, J.J. *Tetrahedron* **1970**, *26*, 2329-2344.
34. Casnati, G.; Quilico, A.; Ricca, A. *Gazz. Chim. Ital.* **1963**, *93*, 349-354.
35. Birch, A.J.; Farrar, K.R. *J. Chem. Soc.* **1963**, 4277-4278.
36. Casnati, G.; Pochini, A. *J. Chem. Soc., Chem. Commun.* **1970**, 1328-1329.
37. Jackson, A.H.; Smith, A.E. *Tetrahedron* **1965**, *21*, 989-1000.
38. Takase, S.; Kawai, Y.; Uchida, I.; Tanaka, H.; Aoki, H. *Tetrahedron Lett.* **1984**, *25*, 4673-4676.

39. Takase, S.; Iwami, M.; Ando, T.; Okamoto, M.; Yoshida, K.; Horiai, H.; Kohsaka, M.; Aoki, H.; Imanaka, H. *J. Antibiotics* **1984**, *37*, 1320-1323.
40. a) Takase, S.; Kavai, Y.; Uchida, I.; Tanaka, H.; Akoi, H. *Tetrahedron* **1985**, *41*, 3037-3048. b) Laws, I.; Mantle, P.G. *Phytochemistry* **1985**, *24*, 1395-1397.
41. Takase, S.; Kawai, Y.; Uchida, I.; Tanaka, H.; Aoki, H. *Tetrahedron* **1985**, *41*, 3037-3048.
42. Scott, P.M.; Merrien, M.; Polonsky, J. *Experientia* **1976**, *32*, 140-142.
43. Ohmomo, S.; Oguma, K.; Ohashi, T.; Abe, M. *Agric. Biol. Chem.* **1978**, *42*, 2387-2389.
44. Ohmomo, S.; Ohashi, T.; Abe, M. *Agric. Biol. Chem.* **1979**, *43*, 2035-2038.
45. a) Bycroft, B.W.; Landon, W. *J. Chem. Soc., Chem. Commun.* **1970**, 168. b) Bycroft, B.W.; Landon, W. *J. Chem. Soc., Chem. Commun.* **1970**, 967-968.
46. Baldwin, J.E. *J. Chem. Soc., Chem. Commun.* **1976**, 734-736.
47. a) Jackson, A.H.; Smith, A.E. *Tetrahedron* **1965**, *21*, 989-1000. b) Casnati, G.; Francioni, M.; Guareschi, A.; Pochini, A. *Tetrahedron Lett.* **1969**, 2485-2487.
48. a) Plieninger, H.; Fischer, R. *Angew. Chem.* **1962**, *74*, 430. b) Robbers, J.E.; Floss, H.G. *Arch. Biochem. Biophys.* **1968**, *126*, 967-969.
49. Kusano, M.; Sotoma, G.; Koshino, H.; Uzawa, J.; Chijimatsu, M.; Fujioka, S.; Kawano, T.; Kimura, Y. *J. Chem. Soc., Perkin Trans. 1* **1998**, 2823-2826.
50. Arai, K.; Kimura, K.; Mushiroda, T.; Yamamoto, Y. *Chem. Pharm. Bull.* **1989**, *37*, 2937-2939.
51. a) Hochlowski, J.E.; Mullally, M.M.; Spanton, S.G.; Whittern, D.N.; Hill, P.; McAlpine, J.B. *J. Antibiotics* **1993**, *46*, 380-386. b) Kane, S.E. *Advances in Drug Research* **1996**, *28*, 181-252.
52. Karwowski, J.P.; Jackson, M.; Rasmussen, R.R.; Humphrey, P.E.; Poddig, J.B.; Kohl, W.L.; Scherr, M.H.; Kadam, S.; McAlpine, J.B. *J. Antibiotics* **1993**, *46*, 374-379.
53. Penn, J.; Mantle, P.G.; Bilton, J.N.; Sheppard, R.N. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1495-1496.
54. a) Wong, S.-M.; Musza, L.L.; Kydd, G.C.; Kullnig, R.; Gillum, A.M.; Cooper, R. *J. Antibiotics* **1993**, *46*, 545-553. b) Fujimoto, H.; Negishi, E.; Yamaguchi, K.; Nishi, N.; Yamazaki, M. *Chem. Pharm. Bull.* **1996**, *44*, 1843-1848.
55. a) Numata, A.; Takahashi, C.; Matsushita, T.; Miyamoto, T.; Kawai, K.; Usami, Y.; Matsumura, E.; Inoue, M.; Ohishi, H.; Shingu, T. *Tetrahedron Lett.* **1992**, *33*, 1621-1624. b) Takahashi, C.; Matsushita, T.; Doi, M.; Minoura, K.; Shingu, T.; Kumeda, Y.; Numata, A. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2345-2353.
56. Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiotics* **1996**, *49*, 832-835.
57. Cui, C.-B.; Kakeya, H..; Osada, H. *Tetrahedron* **1996**, *52*, 12651-12666.
58. Edmondson, S.D.; Danishefsky, S.J. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1138-1140.
59. Edmondson, S.D.; Danishefsky, S.J.; Sepp-Lorenzino, L.; Rosen, N. *J. Am. Chem. Soc.* **1999**, *121*, 2147-2155.
60. Harrison, D.M. *Tetrahedron Lett.* **1981**, *22*, 2501-2504.
61. Cole, R.J.; Cox, R.H. Tremorgen group. *In:* Handbook of Toxic Fungal Metabolites. Academic Press, New York, **1981**, pp. 355-509.
62. Intravenous doses in the range of as little as 5 to 15 µg/kg in sheep or pig have been reported to be tremorgenic [Perera, K.P.W.C.; Day, J.B.; Mantle, P.G.; Rodrigues, L. *Appl. Environm. Microbiol.* **1982**, *43*, 503-508 and references cited therein].
63. Norris, P.J.; Smith, C.C.T.; DeBelleroche, J.; Bradford, H.F.; Mantle, P.G.; Thomas, A.J.; Penny, R.H.C. *J. Neurochem.* **1980**, *34*, 33-42.
64. Yamazaki, M.; Suzuki, S.; Kukita, K. *J. Pharmacobiodyn.*. **1979**, *2*, 119-125.
65. Yamazaki, M.; Fujimoto, H.; Kawasaki, T. *Chem. Pharm. Bull.* **1980**, *28*, 245-254
66. Peterson, D.W.; Bradford, H.F.; Mantle, P.G. *Biochem. Pharmacol.* **1982**, *31*, 2807-2810.
67. Shreeve, B.J.; Patterson, D.S.P.; Roberts, B.A.; MacDonald, S.M. *Vet. Res. Commun.* **1983**, *7*, 155-160.
68. Suzuki, S.; Kikkawa, K.; Yamazaki, M. *J. Pharmacobiodyn.*. **1984**, *7*, 935-942.
69. Nishiyama, M.; Kuga, T. *Japan. J. Pharmacol.* **1986**, *40*, 481-489.

70. Nishiyama, M.; Kuga, T. *Japan. J. Pharmacol.* **1989**, *50*, 167-173.
71. Nishiyama, M.; Kuga, T. *Japan. J. Pharmacol.* **1990**, *52*, 201-208.
72. Hotujac, Lj.; Muftic, R.H.; Filipovic, N. *Pharmacology* **1976**, *14*, 297-300.
73. Selala, M.I.; Daelemans, F.; Schepens, P.J.C. *Drug Chem. Toxicol.* **1989**, *12*, 237-257.
74. Peterson, D.W.; Bradford, H.F.; Mantle, P.G. *Biochem. Pharmacol.* **1982**, *31*, 2807-2810.
75. Selala, M.I.; Laekeman, G.M.; Loenders, B.; Masuku, A.; Herman, A.G.; Schepens, P.J.C. *J. Nat. Prod.* **1991**, *54*, 207-212.
76. Knaus, H.-G.; McManus, O.B.; Lee, S.H.; Schmalhofer, W.A.; Garcia-Calvo, M.; Helms, L.M.H.; Sanchez, M.; Giangiacomo, K.; Reuben, J.P.; Smith III, A.B.; Kaczorowski, G.J.; Garcia, M.L. *Biochemistry* **1994**, *33*, 5819-5828.
77. The G2 synchronously cells are generally more sensitive to G2/M phase inhibitors than the randomly cultured cells.[28]
78. Usui, T.; Kondoh, M.; Cui, C.-B.; Mayumi, T..; Osada, H. *Biochem. J.* **1998**, *333*, 543-548.
79. Kondoh, M.; Usui, T.; Mayumi, T.; Osada, H. *J. Antibiot.* **1998**, *51*, 801-804.
80. Garland, D.L. *Biochemistry* **1978**, *17*, 4266-4272.
81. Himes, R.H.; Kersey, R.N.; Heller-Bettinger, I.; Samson, E. *Cancer Res.* **1976**, *36*, 3798-3802.
82. Schiff, P.B.; Fant, J.; Horwitz, S.B. *Nature* **1979**, *277*, 665-667.
83. Bollag, D.M.; McQueney, P.A.; Zhu, J.; Hensens, O.; Koupal, L.; Liesch, J.; Goetz, M.; Lazarides, E.; Woods, C.M. *Cancer Res.* **1995**, *55*, 2325-2333.
84. Sanz-Cervera, J.F.; Stocking, E.M.; Usui, T.; Osada, H.; Williams, R.M. *Bioorg. Med. Chem.* **2000**, *8*, 2407-2415.
85. Wang, H.; Usui, T.; Osada, H.; Ganesan, A. *J. Med. Chem.* **2000**, *43*, 1577-1585.
86. Klein, I.; Sarkadi, B.; Váradi, A. *Biochim. Biophys. Acta.* **1999**, *1461*, 237-262.
87. Hrycyna, C.A.; Gottesman, M.M. *Drug Resistance Updates* **1998**, *1*, 81-83.
88. Higgins, C.F. *Annu. Rev. Cell Biol.* **1992**, *8*, 67-113.
89. Schneider, E.; Hunke, S. *FEMS Microbiol. Rev.* **1998**, *22*, 1-20.
90. a) Senior, A.E.; al-Shawi, M.K.; Urbatsch, I.L. *FEBS Lett.* **1995**, *377*, 285-289. b) Senior, A.E.; Gadsby, D.C. *Semin. Cancer Biol.* **1997**, *8*, 143-150.
91. Urbatsch, I.L.; Sankaran, B.; Weber, J.; Senior, A.E. J. Biol. Chem. 1995, 270, 19383-19390.
92. Bolhuis, H.; van Veen, H.W.; Poolman, B.; Driessen, A.J.M.; Konings, W.N. *FEMS Microbiol. Rev.* **1997**, *21*, 55-84.
93. Juliano, R.L.; Ling, V. *Biochim. Biophys. Acta* **1976**, *445*, 152-162.
94. Gros, P.; Croop, J.; Housman, D. *Cell* **1986**, *47*, 371-380.
95. Cole, S.P.C.; Bhardwaj, G.; Gerlach, J.H.; Mackie, J.E.; Grant, C.E. Almquist, K.C.; Stewart, A.J.; Kurz, E.U.; Duncan, A.M.V.; Deeley, R.G. *Science* **1992**, *258*, 1650-1654.
96. Biedler, J.L.; Riehm, H.; *Cancer Res.* **1970**, *30*, 1174-1184.
97. An interesting question is whether a patient's chronic exposure to certain xenobiotics, such as long-term use of pharmaceuticals that are not related to cancer therapy but are substrates for a certain ABC transporter, might exert a selective pressure, so that the tumor developing in that patient would overexpress this transporter and be more chemoresistant than a tumor that developed in a patient without such a drug history [Egorin, M.J. *J. Natl. Cancer Inst.* **2000**, *92*, 1628-1629].
98. It is still unclear whether the (over)expression of MDR related transporters is purely drug-induced (e.g. due to regulation of expression at the transcription level or gene amplification), or possibly also as the result of mutations due to the genomic instability of cancer cells and their uncontrolled high rate of cell proliferation (due to one can envisage a relatively high chance of accidental overexpression of MDR genes).
99. Sikic, B.I. *Oncology* **1999**, *13*, 183-187.
100. a) Thiebaut, F.; Tsuruo, T.; Hamada, H.; Gottesman, M.M.; Pastan, I.; Willingham, M.C. *Proc. Natl. Acad. Sci USA* **1987**, *84*, 7735-7738. b) van Helvoort, A.; Smith, A.J.; Sprong, H.; Fritzsche, I.; Schinkel, A.H.; Borst, P.; van Meer, G. *Cell* **1996**, *87*, 507-517.

101. Cordon-Cardo, C.; O'Brien, J.P.; Casals, D.; Rittman-Grauer, L.; Biedler, J.L.; Melamed, M.R.; Bertino, J.R. *Proc. Natl. Acad. Sci USA* **1989**, *86*, 695-698.
102. Rao, V.V.; Dahlheimer, J.L.; Bardgett, M.E.; Snyder, A.Z.; Finch, R.A.; Sartorelli, A.C.; Piwnica-Worms, D. *Proc. Natl. Acad. Sci USA* **1999**, *96*, 3900-3905.
103. Yeh, G.C.; Lopaczynska, J.; Poore, C.M.; Phang, J.M. *Cancer Res.* **1992**, *52*, 6692-6695.
104. Ueda, K.; Okamura, N.; Hirai, M.; Tanigawara, Y.; Saeki, T.; Kioka, N.; Komano, T.; Hori, R. *J. Biol. Chem.* **1992**, *267*, 24248-24252.
105. Ford, J.M. *Eur. J. Cancer* **1996**, *32A*, 991-1001.
106. a) Ferry, D.R.; Russell, M.A.; Cullen, M.H. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 440-445. b) Martin, C.; Berridge, G.; Higgins, C.F.; Callaghan, R. *Br. J. Pharmacol.* **1997**, *122*, 765-771.
107. Borst, P.; Schinkel, A.H. *Eur. J. Cancer* **1996**, *32A*, 985-990.
108. a) Schinkel, A.H.; Smit, J.J.M.; van Tellingen, O.; Beijen, J.H.; Wagenaar, E.; van Deemter, L.; Mol, C.A.A.M.; van der Valk, M.A. Robanus-Maandag, E.C.; te Riele, H.P.J.; Berns, A.J.M.; Borst, P. *Cell* **1994**, *77*, 491-502. b) Schinkel, A.H.; Wagenaar, E.; van Deemter, L.; Mol, C.A.A.M.; Borst, P. *J. Clin. Invest.* **1995**, *96*, 1698-1705. c) Schinkel, A.H.; Mayer, U.; Wagenaar, E.; Mol, C.A.A.M.; van Deemter, L.; Smit, J.J.M.; van der Valk, M.A.; Voordouw, A.C.; Spits, H.; van Tellingen, O.; Zijlmans, J.M.; Fibbe, W.E.; Borst, P. *Proc. Natl. Acad. Sci USA* **1997**, *94*, 4028-4033.
109. Smit, J.J.M.; Schinkel, A.H.; Oude Elferink R.P.J.; Groen, A.K.; Wagenaar, E.; van Deemter, L.; Mol, C.A.A.M.; Ottenhoff, R.; van der Lugt, N.M.T.; van Roon, M.A.; van der Valk, M.A.; Offerhaus, G.J.A.; Berns, A.J.M.; Borst, P. *Cell* **1993**, *75*, 451-465.
110. Lautier, D.; Canitrot, Y.; Deeley, R.G.; Cole, S.P.C. *Biochem. Pharmacol.* **1996**, *52*, 967-977.
111. a) Zaman, G.J.R.; Flens, M.J.; van Leusden, M.R.; de Haas, M.; Mulder, H.S.; Lankelma, J.; Pinedo, H.M.; Scheper, R.J.; Baas, F.; Broxterman, H.J.; Borst, P. *Proc. Natl. Acad. Sci USA* **1994**, *91*, 8822-8826. b) Jedlitschky, G.; Leier, I.; Buchholz, U.; Center, M.; Keppler, D. *Cancer Res.* **1994**, *54*, 4833-4836. c) Zaman, G.J.; Lankelma, J.; van Tellingen, O.; Beijnen, J.; Dekker, H.; Paulusma, C.; Oude Elferink, R.P.; Baas, F.; Borst, P. *Proc. Natl. Acad. Sci USA* **1995**, *92*, 7690-7694. d) Hollo, Z.; Homolya, L.; Hegedus, T.; Sarkadi, B. *FEBS Lett.* **1996**, *383*, 99-104.
112. a) Flens, M.J.; Zaman, G.J.R.; van der Valk, P.; Izquierdo, A.B.; Schroeijers, A.B.; Scheffer, G.L.; de Haas, M.; Meijer, C.J.L.M.; Scheper, R.J. *Am. J. Pathol.* **1996**, *148*, 1237-1247. b) Evers, R.; Zaman, G.J.; van Deemter, L.; Jansen, H.; Calafat, J.; Oomen, L.C.; Oude Elferink, R.P.; Borst, P.; Schinkel, A.H. *J. Clin. Invest.* **1996**, *97*, 1211-1218.
113. a) Wijnholds, J.; Evers, R.; van Leusden, M.R.; Mol, C.A.; Zaman, G.J.; Mayer, U.; Beijnen, J.H.; van der Valk, M.; Krimpenfort, P.; Borst, P. *Nat. Med.* **1997**, *11*, 1275-1279. b) Wijnholds, J.; Scheffer, G.L.; van der Valk, M.; van der Valk, P.; Beijnen, J.H.; Scheper, R.J.; Borst, P. *J. Exp. Med.* **1998**, *188*, 797-808.
114. a) Koike, K.; Kwabe, T.; Tanaka, T.; Toh, S.; Uchiumi, T.; Wada, M.; Akiyama, S.; Ono, M.; Kuwano, M. *Cancer Res.* **1997**, *57*, 5475-5479. b) Cui, Y.; Konig, J.; Buchholz, J.K.; Spring, H.; Leier, I.; Keppler, D. *Mol. Pharmacol.* **1999**, *55*, 929-937. c) Hooijberg, J.H.; Broxterman, H.J.; Kool, M.; Assaraf, Y.G.; Peters, G.J.; Noordhuis, P.; Scheper, R.J.; Borst, P.; Pinedo, H.M.; Jansen, G. *Cancer Res.* **1999**, *59*,2532-2535.
115. a) Kool, M.; van der Linden, M.; de Haas, M.; Scheffer, G.L.; de Vree, J.M.L.; Smith, A.J.; Jansen, G.; Peters, G.J.; Ponne, N.; Scheper, R.J.; Oude Elferink, R.P.J.; Baas, F.; Borst, P. *Proc. Natl. .Acad. Sci. USA* **1999**, *96*, 6914-6919. b) Zeng, H.; Bain, J.L.; Belinsky, M.G.; Kruh, G.D. *Cancer Res.* **1999**, *59*, 5964-5967.
116. Hipfner, D.R.; Deeley, R.G.; Cole, S.P.C. *Biochim. Biophys. Acta* **1999**, *1461*, 359-376.
117. a) Kast, C.; Canfield, V.; Levenson, R.; Gros, P. *Biochemistry* **1995**, *34*, 4402-4411. b) Kast, C.; Canfield, V.; Levenson, R.; Gros, P. *J. Biol. Chem.* **1996**, *271*, 9240-9248.
118. Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. *Biochim. Biophys. Acta* **1999**, *1461*, 347-357.
119. Bakos, E.; Evers, R.; Szakács, G.; Tusnády, G.E.; Welker, E.; Szabó, K.; de Haas, M.; van Deemter, L.; Borst, P.; Váradi, A.; Sarkadi, B. *J. Biol. Chem.* **1998**, *273*, 32167-32175.

120. Ling, V. Cancer Chemother. Pharmacol. **1997**, *40* (Suppl), S3-S8, and references cited therein.
121. Ford, J.M. *Eur. J. Cancer* **1996**, *32A*, 991-1001.
122. a) Gruber, A.; Peterson, C.; Reizenstein, P. *Int. J. Cancer* **1988**, *41*, 224-226. b) Plumb, J.A.; Milroy, R.; Kaye, S.B. *Biochem. Pharmacol.* **1990**, *39*, 787-792. c) Hollt, V.; Kouba, M.; Dietel, M.; Vogt, G. *Biochem. Pharmacol.* **1992**, *43*, 2601-2608.
123. a) Lum, B.L.; Fisher, G.A.; Brophy, N.A.; Yahanda, A.M.; Adler, K.M.; Kaubisch, S.; Halsey, J.; Sicic, B.I. *Cancer* **1993**, *72 (suppl.)*, 3502-3514. b) Raderer, M.; Scheithauer, W. *Cancer* **1993**, *72*, 3553-3563.
124. a) Ford, J.M.; Hait, W.N. *Pharmacol. Rev.* **1990**, *42*, 156-199. B) Hu, X.F.; Martin, T.J. Bewll, D.R.; Luise, M.; Zalcberg, J.R. *Cancer Res.* **1990**, *50*, 2953-2957. c) Osann, K.; Sweet, P.; Slater, L.M. *Cancer Chemother. Pharmacol.* **1992**, *30*, 152-154.
125. a) Boesch, D.; Gaveraux, C.; Jachez, B.; Portier-Manzanedo, A.; Bollinger, P.; Loor, F. *Cancer Res.* **1991**, *51*, 4226-4233. b) Twentyman, P.R.; Bleehen, N.M. *Eur. J. Cancer* **1991**, *27*, 1639-1642. c) Boote, D.; Dennis, I.F.; Twentyman, P.R.; Osborne, R.J.; Laburte, C.; Hensel, S.; Smyth, J.F.; Brampton, M.H.; Bleehen, N.M. *J. Clin. Oncol.* **1996**, *14*, 610-618. d) Lush, R.M.; Meadows, B.; Fojo, A.T.; Kalafsky, G.; Smith, H.T.; Bates. S.; Figg, W.D. *J. Clin. Pharmacol.* **1997**, *37*, 123-128.
126. Dantzig, A.H.; Shepard, R.L.; Cao, J.; Law, K.L.; Ehlhardt, W.J.; Baughman, T.M.; Bumol, T.F.; Starling, J.J. *Cancer Res.* **1996**, *56*, 4171-4179.
127. Hyafil, F.; Vergely, C.; du Vignaud, P.; Grand-Perret, T. *Cancer Res.* **1993**, *53*, 4595-4602.
128. Dale, I.L.; Tuffley, W.; Callaghan, R.; Holmes, J.A.; Martin, K.; Luscombe, M.; Mistry, P.; Ryder, H.; Stewart, A.J.; Charlton, P.; Twentyman, P.R.; Bevan, P. *Br. J. Cancer* **1998**, *78*, 885-892.
129. a) Stewart, A.J.; Mistry, P.; Dangerfield, W.; Okiji, S.; Templeton, D. *Annals of Oncology* **1998**, *9*, 145. b) Mistry, P.; Bootle, D.; Liddle, C.; Loi, R.; Templeton, D. *Annals of Oncology* **1998**, *9*, 148. c) Roe, M.; Folkes, A.; Ashworth, P.; Brumwell, J.; Chima, L.; Hunjan, S.; Pretswell, I.; Dangerfield, W.; Ryder, H.; Charlton, P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 595-600.
130. Martin, C.; Berridge, G.; Mistry, P.; Higgins, C.; Charlton, P.; Callaghan, R. *Br. J. Pharmacol.* **1999**, *128*, 403-411.
131. de Bruin, M.; Miyake, K.; Litman, T.; Robey, R.; Bates, S.E. *Cancer Letters* **1999**, *146*, 117-126.
132. Krishna, R.; Mayer, L.D. *Eur. J. Pharm. Sci.* **2000**, *11*, 265-283.
133. a) van Asperen, J.; van Tellingen, O.; Sparreboom, A.; Schinkel, A.H.; Borst, P.; Nooijen, W.J.; Beijnen, J.H. *Br. J. Cancer* **1997**, *76*, 1181-1183. b) van Asperen, J.; van Tellingen, O.; van der Valk, M.A.; Rozenhart, M.; Beijnen, J.H. *Clin. Cancer Res.* **1998**, *4*, 2293-2297.
134. Most anticancer drugs have a narrow therapeutic index (i.e., toxicity for a tumor as opposed to overall toxicity to the organism). Since bioavailability after oral administration is not only low due to intestinal multidrug transporter activity, but also shows significant inter-patient variation (probably due to differences in transporter expression levels), intravenous administration is currently the most reliable treatment.
135. Schinkel, A.H. *J. Clin. Pharm. Ther.* **1998**, *36*, 9-13.
136. Lum, B.; Gosland, M. *Hematol. Oncol. Clin. North Am.* **1995**, *9*, 319-336.
137. Ouellette, M.; Kündig, C. *Int. J. Antimicrob. Agents* **1997**, *8*, 179-187.
138. van Bambeke, F.; Balzi, E.; Tulkens, P.M. *Biochem. Pharmacol.* **2000**, *60*, 457-470.
139. Bolhuis, H.; Molenaar, D.; Poelarends, G.; van Veen, H.W.; Poolman, B.; Driessen, A.J.M.; Konings, W.N. *J. Bacteriol.* **1994**, *176*, 6957-6964.
140. van Veen, H.W.; Venema, K.; Bolhuis, H.; Oussenko, I.; Kok, J.; Poolman, B.; Driessen, A.J.M.; Konings, W.N. *Proc. Natl. Acad. Sci USA* **1996**, *93*, 10668-10672.
141. Putman, M.; van Veen, H.W.; Degener, J.E.; Konings, W.N. *Mol. Microbiol.* **2000**, *36*, 772-774.
142. van Veen, H.W.; Konings, W.N. Biochim. Biophys. Acta 1998, 1365, 31-36.
143. van Veen, H.W.; Margolles, A.; Putman, M.; Sakamoto, K.; Konings, W.N. *Antonie van Leeuwenhoek* **1999**, *76*, 347-352.
144. Endicott, J.A.; Ling, V. *Annu. Rev. Biochem.* **1989**, *58*, 137-171.

145. van Veen, H.W.; Callaghan, R.; Soceneantu, L.; Sardini, A.; Konings, W.N.; Higgins, C.F. *Nature* **1998**, *391*, 291-295.

146. Gottesman, M.M.; Hrycyna, C.A.; Schoenlein, P.V.; Germann, U.A.; Pastan, I. *Annu. Rev. Genet.* **1996**, *29*, 607-649.

147. van Veen, H.W.; Margolles, A.; Müller, M.; Higgins, C.F.; Konings, W.N. *EMBO J.* **2000**, *19*, 2503-2514.

148. The half-transporter quandary is a subject of intense interest, with the discovery of a number of related mammalian and prokaryotic ABC transporters having this structure. Their study should gain insight in defining the minimal functional unit of ABC transporters, i.e. whether or not a single half-transporter is capable of full function.

149. a) Higgins, C.F.; Gottesman, M.M. *Trends Biochem. Sci.* **1992**, *17*, 18-21. b) Higgins, C.F. *Cell* **1994**, *79*, 393-395. c) Higgins, C.F. *Curr. Biol.* **1994**, *4*, 259-260.

150. a) Raviv, Y.; Pollard, H.B.; Bruggeman, E.P.; Pastan, I.; Gottesman, M.M. *J. Biol. Chem.* **1990**, *265*, 3975-3980. b) Gottesman, M.M.; Pastan, I. *Annu. Rev. Biochem.* **1993**, *62*, 385-427.

151. Bolhuis, H.; van Veen, H.W.; Molenaar, D.; Poolman, B.; Driessen, A.J.M.; Konings, W.N. *EMBO J.* **1996**, *15*, 4206-4212.

152. Molenaar, D.; Bolhuis, H.; Abee, T.; Poolman, B.; Konings, W.N. *J. Bacteriol.* **1992**, *174*, 3118-3124.

153. Doyle, L.A.; Yang, W.; Abruzzo, L.V.; Krogmann, T.; Gao, Y.; Rishi, A.K.; Ross, D.D. *Proc. Natl. Acad. Sci USA* **1998**, *95*, 15665-15670.

154. Allikmets, R.; Schriml, L.M.; Hutchinson, A.; Romano-Spica, V.; Dean, M. *Cancer Res.* **1998**, *58*, 5337-5339.

155. Miyake, K.; Mickley, L.; Litman, T.; Zhan, Z.; Robey, R.; Cristensen, B.; Brangi, M.; Greenberger, L.; Dean, M.; Fojo, T.; Bates, S.E. *Cancer Res.* **1999**, *59*, 8-13.

156. In *Drosophila*, the White half transporter protein dimerizes with Brown for transport of guanine, and with Scarlet for transport of tryptophan. Both amino acids are eye pigment precursors in this species.

157. Scheffer, G.L.; Maliepaard, M.; Pijnenborg, A.C.L.M.; van Gastelen, M.A.; de Jong, M.C.; Schroeijers, A.B.; van der Kolk, D.M.; Allen, J.D.; Ross, D.D.; van der Valk, P.; Dalton, W.S.; Schellens, J.H.M.; Scheper, R.J. *Cancer Res.* **2000**, *60*, 2589-2593.

158. Ross, D.D. *Leukemia.* **2000**, *14*, 467-473.

159. Jonker, J.W.; Smit, J.W.; Brinkhuis, R.F.; Maliepaard, M.; Beijnen, J.H.; Schellens, J.H.M.; Schinkel, A.H. *J. Natl. Cancer Inst.* **2000**, *92*, 1651-1656.

160. Selection of tumor cells for resistance to mitoxantrone frequently results in sublines with decreased drug accumulation showing a non-P-gp, non-MRP phenotype.

161. Chen, Y.N.; Mickley, L.A.; Schwartz, A.M.; Acton, E.M.; Hwang, J.L.; Fojo, A.T. *J. Biol. Chem.* **1990**, *265*, 10073-10080.

162. Dietel, M.; Arps, H.; Lage, H.; Niendorf, A. *Cancer Res.* **1990**, *50*, 6100-6106.

163. Futscher, B.W.; Abbaszadegan, M.R.; Domann, F.; Dalton, W.S. *Biochem. Pharmacol.* **1994**, *47*, 1601-1606.

164. Lee, J.S.; Scala, S.; Matsumoto, Y.; Dickstein, B.; Robey, B.; Zhan, Z.; Altenberg, G.; Bates, S.E. *J. Cell Biochem.* **1997**, *65*, 513-526.

165. Yang, C.J.; Horton, J.K.; Cowan, K.H.; Schneider, E. *Cancer Res.* **1995**, *55*, 4004-4009.

166. Rabindran, S.K.; He, H.; Singh, M.; Brown, E.; Collins, K.I.; Annable, T.; Greenberger, L.M. *Cancer Res.* **1998**, *58*, 5850-5858.

167. Hazlehurst, L.A.; Foley, N.E.; Gleason-Guzman, M.C.; Hacker, M.P.; Cress, A.E.; Greenberger, L.W.; de Jong, M.C.; Dalton, W.S. *Cancer Res.* **1999**, *59*, 1021-1028.

168. Ross, D.D.; Yang, W.; Abruzzo, L.V.; Dalton, W.S.; Schneider, E.; Lage, H.; Dietel, M.; Greenberger, L.; Cole, S.P.; Doyle, L.A. *J. Natl. Cancer Inst.* **1999**, *91*, 429-433.

169. a) Ross, D.D.; Karp, J.; Yang, W.; Gao, Y.; Abruzzo, L.V.; Doyle, L.A. *Blood* **1998**, *92*, 386A. b) Ross, D.D.; Karp, J.E.; Chen, T.T.; Doyle, L.A. *Blood* **2000**, *96*, 365-368.

170. Yang, C.-H.; Schneider, E.; Kuo, M.-L.; Volk, E.L.; Rocchi, E.; Chen, Y.-C. *Biochem. Pharmacol.* **2000**, *60*, 831-837.
171. It has been reported that, besides from increased intestinal (re)uptake, decreased hepatobiliary excretion is also responsible for part of the increased bioavailability of topotecan due to inhibition of Bcrp1.[159]
172. Maliepaard, M.; van Gastelen, M.A.; de Jong, L.A.; Pluim, D.; van Waardenburg, R.C.A.M.; Ruevekamp-Helmers, M.C.; Floot, B.G.J.; Schellens, J.H.M. *Cancer Res.* **1999**, *59*, 4559-4563.
173. Allen, J.D.; Brinkhuis, R.F.; Wijnholds, J.; Schinkel, A.H. *Cancer Res.* **1999**, *59*, 4237-4241.
174. Allen, J.D.; Brinkhuis, R.F.; van Deemter, L.; Wijnholds, J.; Schinkel, A.H. *Cancer Res.* **2000**, *60*, 5761-5766.
175. Litman, T.; Brangi, M.; Hudson, E.; Fetsch, P.; Abati, A.; Ross, D.D.; Miyake, K.; Resau, J.H.; Bates, S.E. *J. Cell Sci.* **2000**, *113*, 2011-2021.
176. Schinkel, A.H.; Mol, C.A.A.M.; Wagenaar, E.; van Deemter, L.; Smit, J.J.M.; Borst, P. *Eur. J. Cancer* **1995**, *31A*, 1295-1298.
177. Rao, V.V.; Dahlheimer, J.L.; Bardgett, M.E.; Snyder, A.Z.; Finch, R.A.; Sartorelli, A.C.; Piwnica-Worms, D. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3900-3905.
178. Chen, W.S.; Luker, K.E.; Dahlheimer, J.L.; Pica, C.M.; Luker, G.D.; Piwnica-Worms, D. *Biochem. Pharmacol.* **2000**, *60*, 413-426.
179. Rabindran, S.K.; Ross, D.D.; Doyle, L.A.; Yang, W.; Greenberger, L.M. *Cancer Res.* **2000**, *60*, 47-50.
180. Hermkens, P.H.H.; Plate, R.; Ottenheijm, H.C.J. *Tetrahedron* **1988**, *44*, 1991-2000.
181. Hino, T.; Kawate, T.; Nakagawa, M. *Tetrahedron* **1989**, *45*, 1941-1944.
182. For a review on total synthesis of fumitremorgins and verruculogens see: Hino, T.; Nakagawa, M. *Heterocycles* **1997**, *46*, 673-704.
183. Hermkens, P.H.H.; Plate, R.; Kruse, C.G.; Scheeren, H.W.; Ottenheijm, H.C.J. *J. Org. Chem.* **1992**, *57*, 3881-3887 and references cited therein.
184. He, H.; Rabindran, S.G.; Greenberger, L.M.; Carter, G.T. *Med. Chem. Res.* **1999**, *9*, 424-437.