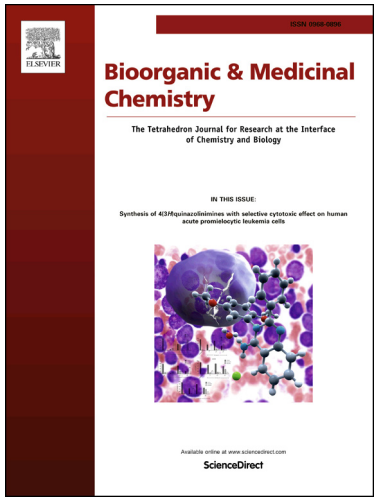

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Lathyrol and epoxylathyrol derivatives: modulation of Cdr1p and Mdr1p drug-efflux transporters of *Candida albicans* in *Saccharomyces cerevisiae* model

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KEYWORDS: Macrocyclic diterpenes; Lathyrane; Multidrug resistance; efflux pumps; Cdr1p; Mdr1p; *Candida albicans, Saccharomyces cerevisiae*

Abstract

Macrocyclic diterpenes were previously found to be able to modulate the efflux pump activity of *Candida albicans* multidrug transporters. Most of these compounds were jatrophanes, but only a few number of lathyrane-type diterpenes was evaluated. Therefore, the aim of this study was to evaluate the ability of nineteen structurally-related lathyrane diterpenes (**1**-**19**) to overcome the drug-efflux activity of Cdr1p and Mdr1p transporters of *C. albicans*, and get some insights on their structure-activity relationships. The transport assay was performed by monitoring Nile Red (NR) efflux in a *Saccharomyces cerevisiae* strain overexpressing the referred efflux pumps from *C. albicans*. Moreover, a chemosensitization assay was performed in order to evaluate the type of interaction between the inhibitory compounds and the antifungal drug fluconazole. Compounds **1**-**13** were previously isolated from *Euphorbia boetica* or obtained by derivatization, and compounds **14**-**19** were prepared by chemical transformations of compound **4**. In the transport assays, compounds **14**-**19** revealed the strongest inhibitory activity of the Cdr1p efflux pump, ranging from 65 to 85%. Concerning Mdr1p efflux pump, the most active compounds were **1**, **3**, **6**, **8**, and **12** (75 to 85%). When used in combination with fluconazole, epoxyboetirane K (**2**) and euphoboetirane N (**18**) revealed synergistic effects in the AD-CDR1 yeast strain, overexpressing the Cdr1p transporter, through their ability to reduce the effective concentration of the antifungal drug by 23- and 52 fold, respectively.

1. Introduction

In the last three decades, invasive fungal infections have considerably increased due to the rising number of immunocompromised patients, the extensive application of drugs such as corticosteroids and immunosuppressants and, paradoxically, medical advances such as organ transplantation or endoscopic techniques.[1] These infections are of major clinical concern, being responsible for substantial morbidity and unacceptably high mortality rates, killing about 1.5 million people every year.[2] The most frequent fungal pathogens are *Candida*, *Cryptococcus*, *Aspergillus*, and *Pneumocystis* spp., which are assumed to be responsible for up to 90% of all reported deaths.[2, 3]

The antifungal agents used in clinical treatments include four major classes of drugs, namely azoles, polyenes, pyrimidine analogs and echinocandins, that mostly target biosynthetic pathways or specific components of the plasma membrane and cell wall.[3, 4] Among them, triazoles have emerged as front-line drugs for the treatment and prophylaxis of many systemic mycoses. In particular, fluconazole is one of the most used antifungals due to its oral availability, great efficacy and reduced side effects.[3, 6] However, extensive and delayed use of azoles in recent years has led to increased tolerance to drugs, and subsequently to the emergence of acquired resistance. Moreover, the clinical azole-resistant isolates not only display decreased susceptibility towards azoles but also show a secondary resistance towards various structurally-unrelated drugs hence displaying multidrug resistance (MDR).[7] In particular, the azole resistance of *Candida albicans* clinical isolates can be caused by several mechanisms that include alteration or overexpression of the target enzyme P450 14-alpha-lanosterol demethylase (P45014DM) involved in ergosterol biosynthesis, change in sterol composition of the plasma membrane, and overexpression of efflux pump proteins that actively efflux drugs out of the cells thus decreasing their effective intracellular concentration.[3, 8] These efflux pump proteins belong to two superfamilies of membrane transporters, namely ATP Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) transporters, which use the hydrolysis of ATP and the plasma membrane electrochemical gradient, respectively, to translocate substrates.[8] Considering the importance of major

antifungal transporters on MDR development, the focus of recent research has been to understand the structure and function of these proteins together with the mechanism of drug extrusion.

One promising approach to circumvent MDR is the development of inhibitors of efflux pumps (also called modulators or chemosensitizers) that when co-administered with the antifungal drug will avoid its efflux thus restoring its cytotoxic concentration.[9, 10] Various new compounds have been established as modulators of MDR pump proteins in pathogenic yeasts, which were obtained either from synthetic or natural sources.[9-12]

Euphorbia species have been a source of several bioactive compounds. In particular, macrocyclic diterpenes with the lathyrane and jatrophane scaffold have been shown to be promising modulators of the human ABCB1 efflux pump in multidrug-resistant cancer cells.[13-16] Furthermore, in previous studies, some macrocyclic diterpenes were evaluated for their potential to inhibit the drug-efflux activity of *C. albicans* Mdr1p and Cdr1p multidrug transporters.[17,18] Several jatrophane diterpenes inhibited both transporters apparently as non-substrates, whereas only three compounds were found to have selectivity for one of these efflux pumps. However, due to the large diversity of structures of that set of compounds, the establishment of structure-activity relationships was not straightforward. In this manner, further studies were necessary in order to optimize plant-derived macrocyclic diterpenes as modulators of efflux pumps belonging to the ABC and MFS superfamilies of transporters. Particularly, regarding lathyrane-type diterpenes very little is known yet. Therefore, the purpose of this study was to evaluate the ability of nineteen structurally-related lathyrane diterpenes, to overcome the drug-efflux activity of Cdr1p and Mdr1p transporters of the pathogenic yeast *C. albicans*, and get some insights on their structure-activity relationships. Moreover, their combinations with the antifungal drug fluconazole were tested in order to obtain evidence of synergistic interactions.

2. Results

2.1. Chemistry

Epoxyboetiranes A (**1**), K (**2**), C – F (**6** – **9**), H – L (**10** – **13**) and epoxylathyrol (**3**) (Fig. 1) were isolated from *E. boetica* aerial parts, or obtained through acylation of **3**.[19] The known macrocyclic diterpene 3,5,15-tri-O-acetyllathyrol[20] (**4**, herein named euphoboetirane A), isolated from the aerial parts of *Euphorbia boetica,* was hydrolyzed affording lathyrol (**5**) that was identified by comparison of its ^{1}H NMR and ^{13}C NMR spectra with those reported in the literature.[21] Several subsequent acylation reactions were performed using a set of different acyl chlorides, yielding six 5-mono-acyl derivatives (**14** – **19**), whose structures were characterized by comparison of their spectroscopic data with those of lathyrol (**5**). Concerning the ester derivatives **14** – **16** and **19**, analysis of ^{1}H-NMR and ^{13}C-NMR spectra showed, as expected, similar data regarding the diterpenic core. When comparing with lathyrol (**5**), remarkable differences in the ^{1}H-NMR spectra were related to H-5 signals that were shifted downfield (max. $\Delta\delta_{H\text{-}5}$ + 1.7 ppm). In the ^{13}C NMR spectra, similar paramagnetic effects at C-5 (α-carbon) were also observed (max. $\Delta\delta_{C\text{-}5}$ + 2.8 ppm). The β-carbon C-6 exhibited significant diamagnetic effects (max. $\Delta\delta_{C\text{-}6}$ = - 3.6 ppm), whereas C-4 was slightly shifted downfield (max. $\Delta\delta_{C\text{-}4}$ + 1.3 ppm). Curiously, a remarkable difference between the ^{13}C NMR spectra of esters **14** – **16** and **19**, and lathyrol (**5**), is the resonance of C-12, which was significantly shifted downfield (max. $\Delta\delta_{C\text{-}12}$ + 10.6 ppm) in the acylated derivatives. As already reported [21], this difference might be due to the conformation adopted by lathyrol (**5**), which decreases the planarity within the enone system, that behaves as an unconjugated ketone as could be evaluated by the C-14 (δ_C 207.1) and C-12 (δ_C 139.9) chemical shifts. Finally, the location of the acyl groups was deduced by the long range correlations between the carbonyl signals and the corresponding H-5 oxymethine protons. The relative stereochemistry of all tetrahedral centers was found to be identical to those of euphoboetirane A.[20]

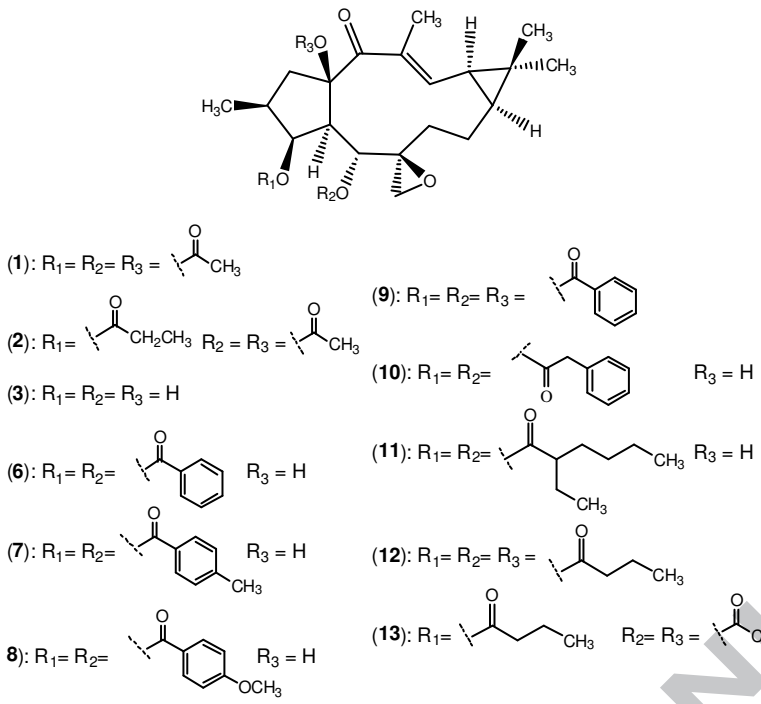
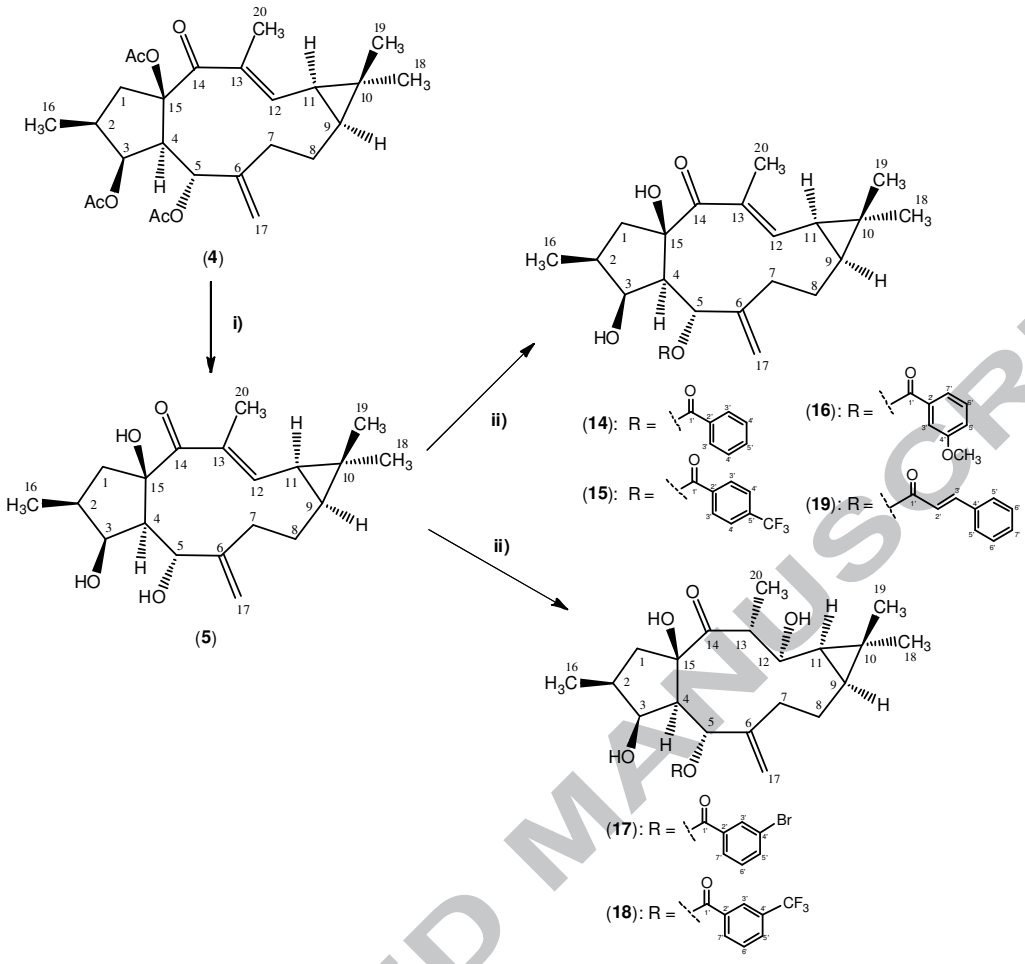


Figure 1. Chemical structures of compounds **1 – 3** and **6 – 13**.

Acylation of lathyrol (**5**) with 3-bromobenzoyl and 3-trifluoromethylbenzoyl chlorides, respectively, afforded compounds **17** and **18,** which underwent further reaction at the β-carbon (C-12) of the α,β-unsaturated ketone, resulting a Michael adduct, having a hydroxyl group at that position. When comparing the NMR spectroscopic data of compounds **17** and **18** to those of already described for the ester derivatives (**14** – **16** and **19**), several differences could be noticed. In the ^{1}H-NMR spectra of compounds **17** and **18** both H-12 and the olefinic methyl (CH_3-20) signal resonances disappeared, while new signals at δ_H 4.73 (bd, J = 9.4 Hz) and δ_H 1.20 (d, J = 7.7 Hz) could be observed. Similarly, these differences were also obvious in the ^{13}C-NMR spectra, which showed the presence of two extra methine carbons at δ_C 74.6 and δ_C 41.5, together with the disappearance of the signals corresponding to the olefinic carbons C-12 and C-13. Moreover, the signal of the carbonyl group was shifted downfield in both compounds ($\Delta\delta_{C\text{-}15} \approx +$ 18.3 ppm), corroborating the absence of the α,β-unsaturated system. These structural features were confirmed by the analysis of ^{1}H-^{1}H-COSY, HMQC and HMBC experiments that allowed the unambiguous assignments of all proton and carbon resonances. The relative configuration of the new tetrahedral stereocenters (C-13 and C-12) of compounds **17** and **18** was deduced through the analysis of their NOESY spectra. Starting from the cyclopropane ring protons H-9α and H-11α, the NOE effects observed between these signals and one of the geminal methyl group at C-10, established the α and β-orientation of CH_3-18 and CH_3-19, respectively. Further cross-peaks between CH_3-19/H-12 and CH_3-20/H-11 dictated the stereochemistry at C-12 and C-13 and the β configuration of these protons.



Scheme 1. Preparation of lathyrol (**5**) derivatives (**14 – 18**).

Reagents and conditions: i) 5% KOH in MeOH (m/v), rt, 24 h, 80% (**5**); ii) acyl chloride, pyridine, rt, 48 h, 14-69%

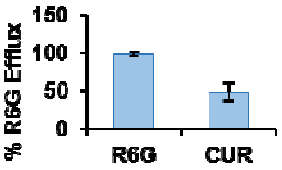
2.2. Biological assays

Compounds **1**-**19** were evaluated for their ability to inhibit the drug-efflux activity of Cdr1p and Mdr1p transporters of *Candida albicans* overexpressed in a *Saccharomyces cerevisiae* strain. The transport assay was performed by monitoring Nile Red (NR) efflux in cells overexpressing the referred efflux pumps. In this assay, compounds were compared to control cells, and when the Nile Red efflux was lower than 60%, the compound was considered to have inhibitory activity (Fig. 2).

Euphoboetiranes J – O (**14** – **19**) revealed the strongest inhibitory activity of Cdr1p efflux pump, ranging from 65 to 85%. Regarding *S. cerevisiae* cells overexpressing Mdr1p, the most active compounds were epoxylathyrol (**3**) > epoxyboetirane J (**12**) > epoxyboetirane E (**6**) > epoxyboetirane D (**8**) > epoxyboetirane A (**1**). All the active compounds were found to be selective. The remaining tested compounds revealed a weak inhibitory activity in both cells lines overexpressing the two types of efflux pumps.

The antifungal property of the macrocyclic diterpenes (**1** – **19**) was evaluated by measuring the growth of control yeast cells (AD1-8u$^-$) and efflux pump-overexpressing cells (CDR1 and MDR1) when exposed to variable concentrations of the compounds for 48 h. The yeast growth in the absence of inhibitor was considered as 100%. The results were expressed as MIC_{80}, the concentration needed to decrease 80% of cells growth (Table 1S, Supporting information). In case of the control yeast cells (AD1-8u$^-$), the compounds did not reveal any significant antifungal property, as demonstrated by their relative resistance index (RI) values that were close to 1. The MIC_{80} values ranging from 498±55 to 1525±167μM, for both sensitive control cells (AD1-8u$^-$) and MDR cells (CDR1 and MDR1), demonstrated the non-toxic nature of the compounds even at higher concentrations, except compounds **3** and **4**, which showed 4-fold lower toxic concentrations for MDR cells (CDR1 and MDR1) as compared to sensitive control cells (AD1-8u$^-$). This indicates the substrate nature of compounds **3** and **4** for the Cdr1p and Mdr1p, as these compounds are transported via MDR transporter overexpressing cells and not by the AD1-8u$^-$ cells. Interestingly, compound **3** was observed to inhibit the Nile Red transport from the AD-MDR1 cells and simultaneously behaved as the substrate of Mdr1p. Concerning the possible mechanism of drug binding, it could be suggested that the Nile Red and compound **3** seem to share the same drug binding pocket of MDR1, undergoing the kinetics of competitive inhibition. When compared to the activity of compound **3** in AD-CDR1 cells, its route of transport and Nile Red efflux did not overlap, indicating the presence of allosteric drug binding pocket for both compounds in CDR1 and thus follow the path of non-competitive kinetics.

The type of interaction of compounds **1** – **19** with the antifungal agent fluconazole was evaluated by the checkerboard method,[22] using control cells (AD1-8u$^-$) and the Cdr1p and Mdr1p-overexpressing cells in presence of different concentrations of fluconazole (4-209 μM) and inhibitors (0.25-400 μM). The results were expressed as the fractional inhibitory concentration index (FICI) and are summarized in Table 2S (Supporting information). FICI values were calculated as the sum of the FIC of each agent (inhibitor and fluconazole); values lower than 0.5 indicated that the compounds exhibited a synergistic interaction with fluconazole.[23] Two compounds, epoxyboetirane K (**2,** FICI = 0.54) and euphoboetirane N (**18,** FICI = 0.52), revealed synergistic effects in the AD-CDR1 yeast strain, by reducing the effective concentration of fluconazole as much as 23- and 52 fold, respectively. In contrast, the other compounds displayed high FICI values (≥ 1.3) in both yeast strains. This synergistic effect in CDR1 could be explained in terms of the presence of allosteric drug binding pockets for fluconazole and compounds **2** and **18**, as fluconazole is a well-known substrate of CDR1 and compounds **2** and **18** are not substrates as shown in Table 2S (Supporting information), but still bind CDR1 to inhibit the efflux of Nile Red.



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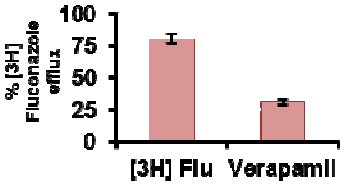

B

Figure 2. Effects of compounds **1**-**19** on Nile Red (NR) efflux by Cdr1p and Mdr1p in *S. cerevisiae* cells overexpressing either the Cdr1p ABC-transporter (AD-CDR1) (**A**) or the Mdr1p MFS-transporter (AD-MDR1) (**B**). Values are the means ± standard deviations (error bars) for three independent experiments. NR was used as the transport substrate at 7 µM, and its efflux was measured by fluorescence. Each inhibitor was used individually at a 10-fold excess over substrate (70 µM). Insets show the inhibition of

rhodamine 6G (R6G) efflux in the presence of curcumin (CUR) taken as positive control for Cdr1p inhibition, and inhibition of [3H] fluconazole ([3H] Flu) efflux in the presence of verapamil taken as positive control for Mdr1p.

3. Discussion

In addition to the well-known potential of macrocyclic diterpenes as strong modulators of human ABCB1 efflux-pump activity in multidrug resistant cancer cells, these compounds have also been identified as promising modulators of efflux pumps in the pathogenic yeast *Candida albicans*. In previous studies, several jatrophane diterpenes and a small group of four lathyranes were evaluated, and some of them showed an interesting profile of activity through their ability to inhibit both Cdr1p and Mdr1p efflux-pumps.[17, 18] Pursuing our research in the field and aiming to specifically increase the data on lathyrane-type diterpenes, herein, compounds **1** - **19** were assayed for their ability to inhibit the Cdr1p and Mdr1p transporters of *C. albicans*. The tested diterpenes can be gathered in three major groups depending on their main structural differences, namely, those derived from epoxylathyrol that are characterized by the existence of the 6,17-epoxy function (**1** – **3**, **6** – **13**), and those derived from lathyrol, which possess an exocyclic ($\Delta^{6,17}$) double bond (**4**, **5**,**14** – **16** and **19**). The acyl derivatives **17** and **18,** which further underwent Michael addition, are characterized by the absence of the endocyclic (Δ^{12}) double bond and by the presence of an extra hydroxyl function at C-13. According to the results, it is interesting to note that the inhibitory activity on both protein transporters seems to be dependent on the type of substituent at C-6. In fact, compounds **14** – **19** ($\Delta^{6,17}$ double bond) revealed to be selective inhibitors of Cdr1p, and, on the other hand, compounds **1**, **3**, **6**, **8** and **12** (6,17-epoxy function) showed selectivity against the Mdr1p-overexpressing yeast strain.

Contrasting with the four lathyranes that were previously evaluated and found to significantly inhibit Nile Red efflux by both transporters,[18] this study has shown that none of the tested compounds

behaved as dual inhibitors. In addition to the different acylation pattern, those lathyranes also differ from compounds **1** – **19** by the presence of a rare 5, 6-epoxy function, suggesting the importance of this structural feature in the inhibitory activity.

When comparing the results obtained for the parent polyalcohol lathyrol (**5**) and its C-5 mono-acyl derivatives (**14** – **19**), in *S. cerevisiae* cells overexpressing Cdr1p, it might be concluded that the presence of an aromatic substituent increases the inhibition activity. Furthermore, the different substituents at the benzene ring in compounds **14** – **19** led to a variable inhibitory potential. The highest activity was obtained for compound **14** that has an unsubstituted benzoyl moiety and displayed 85% inhibition. On the other hand, substitution of the benzene ring with the strong electron withdrawing trifluormethyl group (-CF_3) in **15** seems to have a detrimental effect on the activity (70% inhibition). Similarly, the presence of a cinnamoyl group at C-5 in **19** decreased the activity, as probably due to steric hindrance that may account for a poor interaction with the protein binding sites. Concerning the Michael adducts **17** and **18**, the existence of an extra hydroxyl function at C-13, instead of the double bond, seems to have no significant effect on the activity.

In order to understand the influence of general physicochemical properties on the inhibitory activity, some molecular descriptors were calculated (Table 3S, Supporting information). Analysis of the results suggested the existence of a preferential log *P* value, between 3.11 and 4.16, indicating a significant hydrophobicity related to the inhibitory activity of the ABC-transporter Cdr1p.

In *S. cerevisiae* cells overexpressing the Major Facilitator transporter Mdr1p, several dissimilarities were observed among the results. Surprisingly, epoxylathyrol (**3**) with three hydroxyl groups, and epoxyboetirane J (**12**) with three propanoyl moieties, were the most active compounds, both showing 85% inhibition. Concerning the aroyl derivatives (**6** – **10**), the most active compounds were epoxyboetiranes E (**6**) and D (**8**), which only differ by the presence of the electron donator methoxyl group in *para*-position of the benzoyl moiety of compound **8**. However, the presence of a methyl group

at the same position drastically decreased the inhibitory activity as can be observed with epoxyboetirane C (**7**). It can also be observed that the presence of an extra benzoyl group at C-15 had a detrimental effect on the activity of compound **9** probably due to the high increase of log *P* value (Table 3S, Supporting information). For this epoxylathyrol set of compounds (**1** – **3** and **6** - **13**), no significant correlations could be found among the calculated physicochemical properties and AD-MDR1 inhibitory activity, suggesting that other factors such as the particular structural features of compounds also played a strong role.

4. Conclusions

In summary, this study showed that macrocyclic lathyrane-type diterpenes could be potential inhibitors of the ABC and MFS transporters, and consequently could be used as reversal agents in antifungal resistance. Additionally, this work strengthens our previous studies, corroborating the importance of the acylation pattern in this type of compounds for inhibiting the activity of Cdr1p and Mdr1p efflux pumps. Further structure–activity relationship studies are still needed, together with a deeper understanding of their mechanisms of interaction with this type of efflux pumps in antifungal resistance.

5. Materials and Methods

5.1. General procedures

All solvents were dried according to published methods and distilled prior to use. Benzoyl chloride was obtained from Merck KGaA, Darmstradt, Germany; all other acyl chlorides were obtained from Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinheim, Germany. Flash column chromatography (CC) was performed on silica gel (Merck 9385), or using CombiFlash® Rf200 (Teledyne Isco). Merck silica gel 60 F254 plates were used in analytical TLC, with visualization under UV light (λ 254 and 366 nm) and by spraying with H_2SO_4/MeOH (1:1), followed by heating. For preparative TLC chromatography, 20 x 20 cm x 0.5 mm silica plates were used (Merck 1.05774). NMR spectra were recorded on a Bruker 300 Avance

spectrometer (^{1}H 300 MHz; ^{13}C 75 MHz), using $CDCl_3$, as solvent. Chemical shifts are expressed in δ (ppm) referenced to the solvent used, and the proton coupling constants *J* in hertz (Hz). Spectra were assigned using appropriate COSY, DEPT, HMQC and HMBC sequences. ESI-MS analysis were performed on a triple quadrupole (QT) Micromass Quattro Micro AP1 mass spectrometer, with an ion source set in a positive ESI ionization mode. All tested compounds were purified to ≥ 95% purity as determined by HPLC and NMR spectroscopy. All the compounds were dissolved in dimethyl sulfoxide (DMSO) to perform the biological assays.

5.2. Compounds tested

Nineteen lathyrane-type diterpenes, whose structures are presented in Fig. 1, were tested: epoxyboetiranes A (**1**), K (**2**), C – F (**6** – **9**), H – L (**10** – **13**), epoxylathyrol (**3**), lathyrol (**5**), euphoboetiranes A (**4**) and J – O (**14** – **19**). Compound **1** and **4** were isolated from *E. boetica* aerial parts, and compounds **6** – **13** were obtained through acylation of **3**, as previously described.[19] Compounds **5** and **14** – **19** were prepared as described below.

5.3. Hydrolysis of euphoboetirane A

Compound **4** (450 mg) in MeOH/KOH (5%) was stirred for 24 h at room temperature. The reaction was worked up by dilution with water (11 mL) and extraction with EtOAc. After drying (Na_2SO_4) and removal of the solvent, 250 mg (yield 80 %) of lathyrol (**5**) were obtained as an amorphous white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.97 (1H, d, *J* = 10.4 Hz, H-12), 5.07 (1H, s, H-17b), 4.92 (1H, s, H-17a), 4.51 (1H, s, 15-OH), 4.39 (1H, d, *J* = 3.6 Hz, H-5), 4.32 (1H, t, *J* = 3.2 Hz, H-3),3.44 (1H, d, *J* = 3.6 Hz, 3-OH), 3.36 (1H, d, *J* = 4.0 Hz, 5-OH), 2.74 (1H, dd, *J* = 14.8 and 9.7 Hz, H-1α), 2.47 (1H, m, H-7a), 2.21 (1H, t, *J* = 3.2 Hz, H-4), 2.13 (1H, m, H-2), 1.93 (3H, s, CH_3-20), 1.80 (1H, m, H-8a), 1.74 (1H, m, H-1β), 1.64 (1H, m, H-7b), 1.35 (1H, dd, *J* = 10.0 and 8.4 Hz, H-11), 1.16 (3H, d, *J* = 6.8 Hz, CH_3-16), 1.13 (3H, s, CH_3-19), 1.11 (3H, s, CH_3-18), 1.06 (1H, m, H-8b), 1.05 (1H, m, H-9) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.1 (C-14), 147.9 (C-6), 139.9 (C-12), 137.2 (C-13), 111.1 (C-17), 88.0 (C-15), 76.7

(C-3), 69.8 (C-5), 53.3 (C-4), 46.9 (C-1), 38.2 (C-2), 35.0 (C-9),33.8 (C-7), 28.7 (C-18), 26.1 (C-11), 24.1 (C-10), 23.4 (C-8), 15.6 (C-19), 14.0 (C-16), 13.8 (C-20) ppm. ESIMS *m/z* 335.1 $[M + H]^+$.

5.4. General preparation of euphoboetiranes J - K

A solution of lathyrol (**5**, 1 eq) in dry pyridine (2 mL) was stirred for 5 min at room temperature before addition of the suitable chloride (3 eq). The mixture was stirred for 48 h at room temperature. The reaction mixture was concentrated under vacuum at 40 ºC, and the obtained residue was purified by flash column chromatography (silica gel, *n*-hexane/ethyl acetate; 1:1) and preparative TLC (*n*-hexane/ethyl acetate, 4:1).

Euphoboetirane J (**14**) was obtained from reaction of **5** (30 mg) with benzoyl chloride (38 mg, 0.029 mmol). The residue was purified to afford 22 mg (0.050 mmol, yield 56 %) of an amorphous white powder.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03 (2H, m, H-3'), 7.57 (1H, m, H-5´), 7.44 (2H, m, H-4'), 7.00, (1H, brs, H-12) 6.07 (1H, d, *J* = 10.1 Hz, H-5), 4.95 (2H, s, H-17), 4.16 (1H, br s, H-3), 3.07 (1H, m, H-1α), 2.58 (1H, dd, *J* = 10.0, 2.7 Hz, H-4), 2.25 (1H, m, H-7a), 2.16 (1H, m, H-2),1.96 (1H, m, H-7b), 1.92 (1H, m, H-8a), 1.82 (3H, s, CH_3-20), 1.68 (1H, dd, *J* = 14.5, 10.2 Hz, H-1β), 1.54 (1H, m, H-8b), 1.44 (1H, dd, *J* = 11.5, 8.6 Hz, H-11), 1.25 (1H, m, H-9), 1.21 (3H, s, CH_3-19),1.16 (3H, s, CH_3-18), 1.11 (3H, d, *J* = 6.8 Hz, CH_3-16) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.0 (C-14), 166.9 (C-1'), 149.9 (C-12), 144.2 (C-6), 135.3 (C-13), 133.5 (C-5´), 130.0 (C-2´), 129.9 (C-3´), 128.6 (C-4´), 114.5 (C-17), 88.4 (C-15), 78.8 (C-3), 71.7 (C-5), 54.2 (C-4), 49.2 (C-1), 37.6 (C-2, C-7), 36.3 (C-9), 28.9 (C-18), 28.3 (C-11), 25.8 (C-10), 21.4 (C-8), 16.2 (C-19), 14.5 (C-16), 13.3 (C-20) ppm; ESIMS *m/z* 439.1 $[M + H]^+$.

Euphoboetirane K (**15**) was obtained from reaction of **5** (40 mg) with 4-trifluoromethylbenzoyl chloride (75 mg, 0.360 mmol). The residue was purified to afford 42 mg (0.083 mmol, yield 69 %) of an amorphous white powder.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (2H, d, *J* = 8.1 Hz, H-3'), 7.69 (2H, d, *J* = 8.2 Hz, H-4'), 6.85 (1H, brs, H-12), 6.09 (1H, d, *J* = 9.8 Hz, H-5), 5.00 (1H, s, H-17a), 4.95 (1H, s, H-17b), 4.15 (1H, br s, H-3), 3.02 (1H, dd, *J* = 14.0, 10.5 Hz, H-1α), 2.60 (1H, dd, *J* = 9.8, 2.9 Hz, H-4), 2.20 (2H, m, H-2 / H-7a), 1.99 (1H, m, H-8a), 1.90 (1H, m, H-7b), 1.85 (3H, s, CH_3-20), 1.64 (1H, m, H-1β), 1.54 (1H, m, H-8b), 1.44 (1H, dd, *J* = 10.8, 8.4 Hz, H-11), 1.26 (1H, m, H-9), 1.22 (3H, s, CH_3-19), 1.16 (3H, s, CH_3-18), 1.11 (3H, d, *J* = 6.8 Hz, CH_3-16) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.9 (C-14), 165.4 (C-1'), 148.8 (C-12), 135.4 (C-13), 135.0 (C-2'), 133.3 (C-5'), 130.3 (C-3') 125.6 (C-4'), 121.8 (CF_3-5'), 114.6 (C-17), 88.2 (C-15), 78.6 (C-3), 72.6 (C-5), 53.6 (C-4), 48.8 (C-1), 37.7 (C-2/C-7), 36.1 (C-9), 28.9 (C-18), 28.2 (C-11), 25.7 (C-10), 21.3 (C-8), 16.4 (C-19), 14.5 (C-16), 13.5 (C-20) ppm; ESIMS *m/z* 507.0 $[M + H]^+$.

Euphoboetirane L (**16**) was obtained from reaction of **5** (40 mg) with 3-methoxybenzoyl chloride (61 mg, 0.56 mmol). The residue was purified by to give 36 mg (0.078 mmol, yield 65 %) of an amorphous white powder.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62 (1H, dt, *J* = 7.8, 1.2 Hz, H-7'), 7.55 (1H, dd, *J* = 2.6, 1.5 Hz, H-3'), 7.10 (1H, ddd, *J* = 8.4, 2.7, 1.0 Hz, H-5'), 7.03 (1H, m, H-12), 6.06 (1H, d, *J* = 10.1 Hz, H-5), 4.95 (2H, s, H-17), 4.16 (1H, br s, H-3), 3.84 (3H, s, OCH_3-4'), 3.05 (1H, m, H-1α), 2.58 (1H, dd, *J* = 10.1, 2.7 Hz, H-4), 2.20 (2H, m, H-2/ H-7a), 1.99 (1H, m, H-8a) 1.82 (3H, s, CH_3-20), 1.67 (1H, m, H-1β), 1.58 (1H, m, H-8b), 1.44 (1H, dd, *J* = 11.5, 8.6 Hz, H-11), 1.26 (1H, m, H-9), 1.22 (3H,s, CH_3-19), 1.17 (1H, m, H-7b), 1.16 (3H, s, CH_3-18), 1.10 (3H, *J* = 6.8 Hz, CH_3-16) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.0 (C-14), 163.2 (C-1'), 159.7, (C-4'), 147.3 (C-12), 144.2 (C-6), 135.3 (C-13), 131.2 (C-2'), 129.6 (C-6'), 122.3 (C-7'), 119.8 (C-5'), 114.7 (C-17), 114.5 (C-5'), 87.0 (C-15), 78.8 (C-3), 70.7 (C-5), 55.6 (C-8'), 53.6 (C-4), 49.2 (C-1), 37.6 (C-2, C-7), 36.3 (C-9), 28.9 (C-18), 28.3 (C-11), 25.8 (C-10), 21.5 (C-8), 16.2 (C-19), 14.5 (C-16), 13.3 (C-20) ppm; ESIMS *m/z* 469.1 $[M + H]^+$.

Euphoboetirane M (**17**) was obtained from reaction of **5** (40 mg) with 3-bromobenzoyl chloride (79 mg, 0.36 mmol). The residue was purified to afford 13 mg (0.024 mmol, yield 21 %) of an amorphous white powder.

^{1}H NMR (300 MHz, $CDCl_3$) δ), 8.12 (1H, t, *J* = 1.8 Hz, H-3'), 7.94 (1H, dt, *J* = 8.1, 1.5 Hz, H-7'), 7.69 (1H, ddd, J = 8.0, 2.0, 1.1 Hz, H-5'), 7.31 (1H, t, *J* = 7.9 Hz, H-6'), 5.69 (1H, d, *J* = 11.1 Hz, H-5), 5.05 (1H, s, H-17a), 4.85 (1H, s, H-17b), 4.73 (1H, bd, *J* = 9.4 Hz, H-12), 3.87 (1H, t, *J* = 3.3 Hz, H-3), 3.05 (1H, dd, *J* = 11.1, 3.4 Hz, H-4), 2.72 (1H, m, H-13), 2.51 (1H, m, H-7β), 2.15 (1H, m, H-7α), 1.94 (3H, m, H-2 / H-8a / H-8b), 1.82 (2H, m, H-1α / H-1β), 1.20 (3H, d, *J* = 7.7 Hz, CH_3-20), 1.13, (3H, s, CH_3-19), 1.05 (3H, s, CH_3-18), 1.03 (3H, d, *J* = 6.6 Hz, CH_3-16), 0.64 (1H, m, H-9), 0.53 (1H, d, *J* = 9.1 Hz, H-11) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 220.5 (C-14), 166.5 (C-1'), 147.1 (C-6), 136.4 (C-5'), 132.9 (C-3'), 131.8 (C-2'), 130.1 (C-6'), 128.6 (C-7'), 122.6 (C-4'), 116.2 (C-17), 87.0 (C-15), 75.0 (C-3), 74.6 (C-12), 71.1 (C-5), 55.0 (C-4), 44.1 (C-1), 41.5 (C-13), 38.2 (C-7), 37.7 (C-2), 30.3 (C-9), 29.1 (C-18), 26.5 (C-11), 23.0 (C-8), 15.5 (C-10/C-19), 13.5 (C-16), 13.1 (C-20) ppm; ESIMS *m/z* 535.0 $[M + H]^+$, 516.9 $[M - H_2O + H]^+$.

Euphoboetirane N (**18**) was obtained from reaction of **5** (30 mg) with 3-trifluoromethylbenzoyl chloride (55 mg, 0.26 mmol). The residue was purified to afford 28 mg (0.054 mmol, yield 60 %) of an amorphous white powder.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (1H, s, H-3'), 8.19 (1H, d, *J* = 7.8 Hz, H-7'), 7.83 (1H, d, *J* = 7.8 Hz, H-5'), 7.58 (1H, t, *J* = 7.8 Hz, H-6'), 5.73 (1H, d, *J* = 11.1 Hz, H-5), 5.06 (1H, s, H-17a), 4.86 (1H, s, H-17b), 4.74 (1H, bd, *J* = 9.4 Hz, H-12), 3.88 (1H, t, *J* = 3.3 Hz, H-3), 3.08 (1H, dd, *J* = 11.1, 3.4 Hz, H-4), 2.73 (1H, qd, *J* = 7.7, 1.5 Hz, H-13), 2.51 (1H, dd, *J* = 13.5, 6.3 Hz, H-7β), 2.17 (1H, m, H-7α), 1.95 (3H, m, H-2/ H-8a/ H-8b), 1.83 (2H, m, H-1α, H-1β), 1.20 (3H, d, *J* = 7.7 Hz, CH_3-20), 1.15 (3H, s, CH_3-19), 1.05 (3H, s, CH_3-18), 1.03 (3H, s, CH_3-16), 0.65 (1H, m, H-9), 0.54 (1H, d, *J* = 9.1 Hz, H-11) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 220.6 (C-14), 166.5 (C-1'), 147.1 (C-6), 133.2 (C-7'), 131.4 (C-2'), 130.8 (C-5'), 129.3 (C-3'), 126.9 (C-3'), 121.9 (CF_3-4'), 116.2 (C-17), 87.0 (C-15), 75.0 (C-3), 74.6 (C-12), 71.3 (C-5), 54.9 (C-4), 44.1 (C-1), 41.5 (C-13), 38.3 (C-7), 37.7 (C-2), 30.3 (C-9), 29.1 (C-18), 26.5 (C-11), 23.0 (C-8), 15.5 (C-10/C-19), 13.5 (C-16), 13.1 (C-20) ppm; ESIMS *m/z* 524.0 $[M]^+$ (not detected), 507.0 $[M - H_2O + H]^+$.

Euphoboetirane O (**19**) was obtained from reaction of **5** (40 mg) with cinnamoyl chloride (60 mg, 0.36 mmol). The residue was purified by flash column chromatography (silica gel, CH_2Cl_2/MeOH; 99:1) and preparative TLC *n*-hexane/ethyl acetate; 4:1) to afford 8 mg (0.017 mmol, yield 14 %) of an amorphous white powder.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72 (1H, d, *J* = 15.9 Hz, H-3'), 7.51 (2H, m, H-6'), 7.38 (3H, m, H-5', H-7'), 7.06 (1H, br s, H-12), 6.44 (1H, d, *J* = 15.9 Hz, H-2'), 5.96 (1H, d, *J* = 10.4 Hz, H-5), 4.95 (1H, s, H-17a), 4.91 (1H, s, H-17b), 4.14 (1H, t, *J* = 3.1 Hz, H-3), 3.11 (1H, m, H-1α), 2.53 (1H, dd, *J* = 10.4, 2.5 Hz, H-4), 2.19 (1H, m, H-2, H-7a), 1.97 (1H, m, H-8a), 1.93 (1H, m, H-7b), 1.78 (3H, s, CH_3-20), 1.66 (1H, m, H-1β), 1.44 (1H, dd, *J* = 11.5, 8.5 Hz, H-11), 1.24 (1H, m, H-9), 1.20 (3H, s, CH_3-19), 1.16 (3H, s, CH_3-18), 1.14 (1H, m, H-8b), 1.12 (3H, s, CH_3-16) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.4 (C-14), 167.9 (C-1'), 150.5 (C-12), 146.4 (C-3'), 144.3 (C-6), 135.3 (C-13), 134.2 (C-4'), 130.8 (C-7'), 129.1 (C-5'), 128.4 (C-6'), 117.6 (C-2'), 114.7 (C-17), 89.3 (C-15), 79.0 (C-3), 71.0 (C-5), 54.6 (C-4), 49.6 (C-1), 37.4 (C-2, C-7), 36.4 (C-9), 29.0 (C-18), 28.5 (C-11), 25.9 (C-10), 21.9 (C-8), 16.3 (C-19), 14.6 (C-16), 13.2 (C-20) ppm; ESIMS *m/z* $[M + H]^+$ 465.1.

5.5. Biological assays

5.5.1. Yeast strains and growth media

The yeast strains used in this study, AD1-8u$^-$, AD-CDR1 and AD-MDR1[24] were cultured in yeast extract peptone-dextrose (YEPD) broth (BIO101; Biomedical Life Systems, Inc., Vista, CA, USA) at 30 °C. For agar plates, 2.5% (w/v) Bacto agar (Difco, BD Biosciences, Franklin, NJ, USA) was added to the medium. All strains were stored as frozen stocks with 15% glycerol at -80 °C. Before each experiment, cells were freshly revived on YEPD plates from the stock.

5.5.2. Reagents and media

Fluconazole was obtained from HiMedia (Mumbai, India). Agar medium was purchased from Difco, BD Biosciences (Franklin Lakes, NJ, USA). Nile Red and other molecular-grade chemicals were obtained from

Sigma Chemical Co. (St. Louis, MO, USA). All routine chemicals were obtained from Qualigens (Mumbai, India) and were of analytical grade.

5.5.3. Transport assays

Transport assays were performed by monitoring Nile Red (NR) efflux in cells overexpressing Cdr1p (AD-CDR1) or Mdr1p (AD-MDR1) by flow cytometry with a FACsort flow cytometer (Becton-Dickinson Immunocytometry Systems). Briefly, cells with an OD of 0.1 ($1.2x10^6$ cells) at 600nm were inoculated and allowed to grow at 30 °C under shaking, until the OD_{600} reached 0.25. The cells were then harvested and resuspended in the medium containing one part of YEPD and two parts of water to make a 5% cell suspension ($O.D_{600}$ is $3.1x10^6$ cells. NR was added to a final concentration of 7 µM, and the cells were incubated at 30 °C for 30 min in absence or presence of each inhibitor at a concentration 10-fold higher than substrate (70 µM). The cells were then harvested, washed twice and the cell sample was prepared by diluting the harvested cells in water. The analysis of Nile Red accumulation was performed using the CellQuest software (Becton Dickinson Immunocytometry Systems), where 10 000 cells were analyzed in the acquisition by the FACS cytometer and the result was expressed as the rate of the efflux of the drug from the yeast cells

5.5.4. Growth inhibition and sensitization to fluconazole (FLC)

Yeast cells (10^4) were seeded into 96-well plates in either absence or presence of varying concentrations of inhibitors (0.25-400 µM), and grown for 48 h at 30 °C. The MIC_{80} values and growth inhibition were determined by measuring the optical density of each strain. Growth in the absence of any inhibitor was considered as 100%, and the concentration producing 80% growth inhibition was taken as the MIC_{80} value; the resistance index (RI) was calculated as the ratio between the MIC_{80} values determined for the strain overexpressing either Cdr1p (AD-CDR1) or Mdr1p (AD-MDR1) relative to that of the control strain (AD1-8u$^-$). The interaction of the respective inhibitors with FLC was evaluated by the checkerboard method recommended by the CLSI (formerly NCCLS), and was expressed as the fractional inhibitory concentration index (FICI). The ranges of concentrations used were 4-209 µM for fluconazole, and 0.25-400 µM for the

inhibitors. FICI values were calculated as the sum of the FICs of each agent (FLC and inhibitors). The FIC of each agent was calculated as the MIC of the agent in combination divided by the MIC of the agent alone.

5.6. Statistical analysis

Data are the means ± SD from duplicate samples of at least three independent experiments. Differences between the mean values were analyzed by Student's t test, and the results were considered as significant when $p < 0.05$.

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Supplementary data

Supplementary data related to this article can be found, in the online version.

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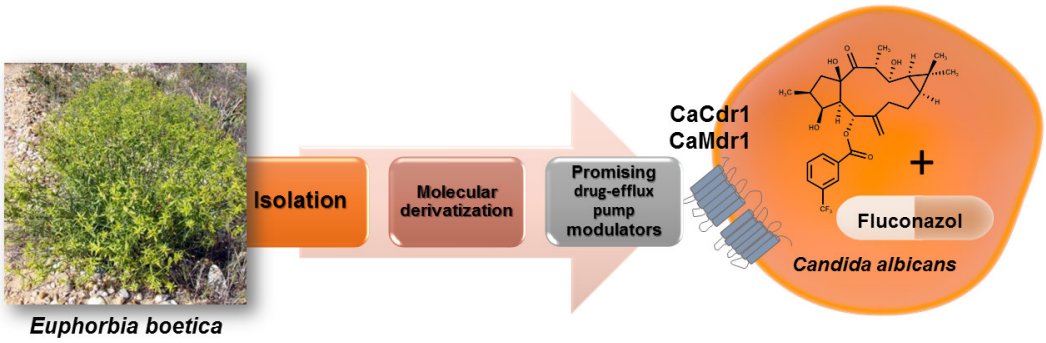
Legends of Figures and Schemes

Figure 1. Chemical structures of compounds **1 – 3** and **6 – 13**.

Figure 2. Effects of compounds **1**-**19** on Nile Red (NR) efflux by Cdr1p and Mdr1p in *S. cerevisiae* cells overexpressing either the Cdr1p ABC-transporter (AD-CDR1) (**A**) or the Mdr1p MFS-transporter (AD-MDR1) (**B**). Values are the means ± standard deviations (error bars) for three independent experiments. NR was used as the transport substrate at 7 µM, and its efflux was measured by fluorescence. Each inhibitor was used individually at a 10-fold excess over substrate (70 µM). Insets show the inhibition of rhodamine 6G (R6G) efflux in the presence of curcumin (CUR) taken as positive control for Cdr1p inhibition, and inhibition of [3H] fluconazole ([3H] Flu) efflux in the presence of verapamil taken as positive control for Mdr1p.

Scheme 1. Preparation of lathyrol (**5**) derivatives (**14 – 18**).

Graphical Abstract



Highlights

- Nineteen diterpenes were evaluated as CaCdr1p and CaMdr1p modulators.
- Some of the compounds revealed strong inhibitory activity of the CaCdr1p efflux pump.
- Some of the modulators synergistically interact with fluconazole.
- Substitution patterns in the molecule play important role in reversal activity.