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Synthesis, anti-fungal activity evaluation and QSAR studies on podophyllotoxin derivatives

K. Anil Kumar[1], Sanjay Kumar Singh[1], B. Siva Kumar[1] and Mukesh Doble[2*]

[1] *Department of Chemistry, Sri Sathya Sai University, Prashanthi Nilayam, 515134 Andhra Pradesh, India*

[2] *Department of Biotechnology, IIT, Madras -600036, India*

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Abstract: The anti-fungal and cytotoxic activites of podophyllotoxin and seven C-4 substituted podophyllotoxin ester derivatives, viz: trans-cinnamyl, cis-cinnamyl, o-methoxy cinnamyl, dimethyl acrylyl, p-methoxy phenyl acetyl, 3,4-dimethoxy phenyl acetyl and 2,5-dimethoxy phenyl acetyl esters were evaluated on four fungi, viz: *Macrophomina phaseolina, Fusarium oxysporum, Myrrothecium verrucarria and Asperigillus candidus,* The podophyllotoxin derivatives were synthesised and their structures were elucidated. Quantitative structure activity relationships were developed between the activity of these compounds against the four fungi and molecular descriptors. The linear regression models developed had one to two descriptors. For all the cases the r^2 was in the range of 0.73 to 0.96, indicating good data fit and q^2 was in the range of 0.60 to 0.68, indicating that the predictive capabilities of the models were acceptable. Solvent accessible surface area (namely the partial positive solvent-accessible surface area), $A \log P$, highest occupied molecular orbital and conformational energy were identified as important descriptors.

Keywords: Podophyllotoxin esters, quantitative structure activity relationships, anti fungal and cancer, solvent accessible surface area

1 Introduction

The discovery that podophyllotoxin (Figure 1) is a potent inhibitor of microtubule assembly has led to considerable work to study its clinical efficacy. Attempts to use it in the treatment of human neoplasia were mostly unsuccessful due to complicated side effects

* E-mail: mukeshd@iitm.ac.in

that included damage to normal tissues [1, 2]. Later more potent and less toxic anticancer agents such as etoposide and teniposide, the semisynthetic glucosidic cyclic acetals of epipodophyllotoxin, were synthesised [3]. The benzaldehyde condensation product of 4'- demethyl podophyllotoxin glucopyronoside and podophyllinic acid ethyl hydrazide was found to have pronounced antimitotic effect. Over the years a number of podophyllotoxin derivatives have been prepared and the list of derivatives prepared has been reviewed in "Podophyllotoxins: Current status and recent developments" [4]. Two moieties of epipodophyllotoxin have been linked at the C4-position to provide novel bisepipodophyllotoxin analogues. Most of these analogues have exhibited in vitro anticancer activity against different human tumour cell lines [5]. In general, these C(4)-modified new derivatives exhibited superior activity profiles, particularly against drug-resistant cell lines, to those of etoposide, hence our focus has also been in synthesizing the C(4) modified derivatives.

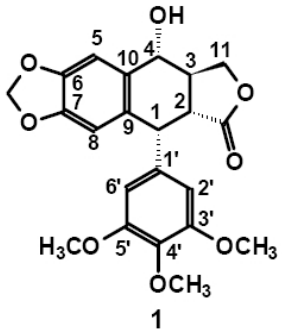

Fig. 1 Structure of podophylotoxin.

Several podophyllotoxin-related ligands have been shown to possess significant antifungal activity against a number of filamentous fungi and structure-activity studies of these compounds indicated that this action is dependent on the groups present at the 4 and 4' positions of the skeleton [6]. The therapeutic value of podophyllotoxins as mitosis inhibitors has medicinal applications including use as an anti-malarial agent [7] and as an anti-fungal agent [8, 9]. Since the α, β - unsaturated carbonyl moiety acts as a potent cytotoxic system, due to its ability to act as a Michael acceptor, seven ester derivatives of podophyllotoxin having this moiety have been synthesised. These derivatives were namely, trans-cinnamyl ester, cis-cinnamyl ester, o-methoxy cinnamyl ester, dimethyl acrylyl ester, 3, 4-dimethoxy phenyl acetyl ester, 2, 5-dimethoxy phenyl acetyl ester and 4-methoxy phenyl acetyl ester.

The prediction of biological properties of organic compounds is one of the main objectives of the method based on quantitative structure-activity relationships (QSAR). The success of this method is very much dependent on appropriate characterisation of the molecular structure and the selection of the structural descriptors. Some structure activity relationship studies have been carried out using several podophyllotoxin analogues, showing that the core structure of deoxypodophyllotoxin is responsible for the cytotoxic-

ity. The extra methoxy group (6-methoxypodophyllotoxin) on the 6-position does not significantly change the *in vitro* cytotoxicity compared to podophyllotoxin. Also the methyl group on the 4'-position of the pendent ring has little effect on the cytotoxicity. QSAR studies on epipodophyllotoxin dimers indicate that GI (50) activity is strongly dependent on structural and thermodynamic properties [10]. QSAR models for 157 epipodophyllotoxins have been reported using multiple topological descriptors of chemical structures, including molecular connectivity indices (MCI) and molecular operating environment descriptors [11]. *In vitro* growth inhibitory activity of 23 podophyllotoxin derivatives against leukemia cells was performed using comparative molecular field analysis and the authors concluded that the B ring is an important part of the activity [12]. Another theoretical study indicated that the C-4 is the position to be used for effective modification and the B ring and E ring are important active centers [13]. Some quinolones with similar electronic construction to podophyllotoxin may have antitumor activity.

The literature has examples of QSAR studies on anti fungal compounds. Two novel structural descriptors namely, lone-pair electrons index (LEI) and molecular volume index (MVI) were found to be effective in developing a QSAR relation on 24 heterocyclic nitrogen-containing compounds against miracidium in liquid, 19 benzyl alcohols against *aspergillus niger* and 50 substituted phenols against *tetrahymena pyriformis* [14]. Rungta *et al.* [15] have considered several structural descriptors to develop a QSAR relationship to predict antifungal activity against *Fusarium* and *Aspergillus* species. Biological activity of 24 chlorinated aliphatic hydrocarbons has been studied in the mold *Aspergillus nidulans* and QSAR analysis indicated that toxic effects induced by these compounds are mainly dependent of steric factors, such as molar refractivity and ease with which they accept electrons, represented by LUMO (energy of the lowest unoccupied molecular orbital) [16]. Wiktorowicz *et al.* [17] have studied the quantitative structure-activity relationships of a series of imidazole derivatives as potential new antifungal drugs. Prabhakar *et al.* [18] have synthesised 2,3,4-substituted thiazolidines and studied their antifungal activity against *Candida albicans, Cryptococcus neoformans, Tricophyton mentagrophyte* and *Aspergillus fumigatus* and developed a QSAR based on the structural descriptors.

In this paper the development of quantitative structure activity relationships for these podophyllotoxins for four fungi namely, *Macrophomina phaseolina, Fusarium oxysporum, Myrthecium verrucarri* and *Aspergillus candidus* is reported.

2 Experimental

The compounds synthesised and characterised in the current work were trans-cinnamyl, cis-cinnamyl, o-methoxy cinnamyl, dimethyl acrylyl, para methoxy phenyl acetyl, 3, 4-dimethoxy phenyl acetyl and 2, 5-dimethoxy phenyl acetyl esters of podophyllotoxin (Figure 2).

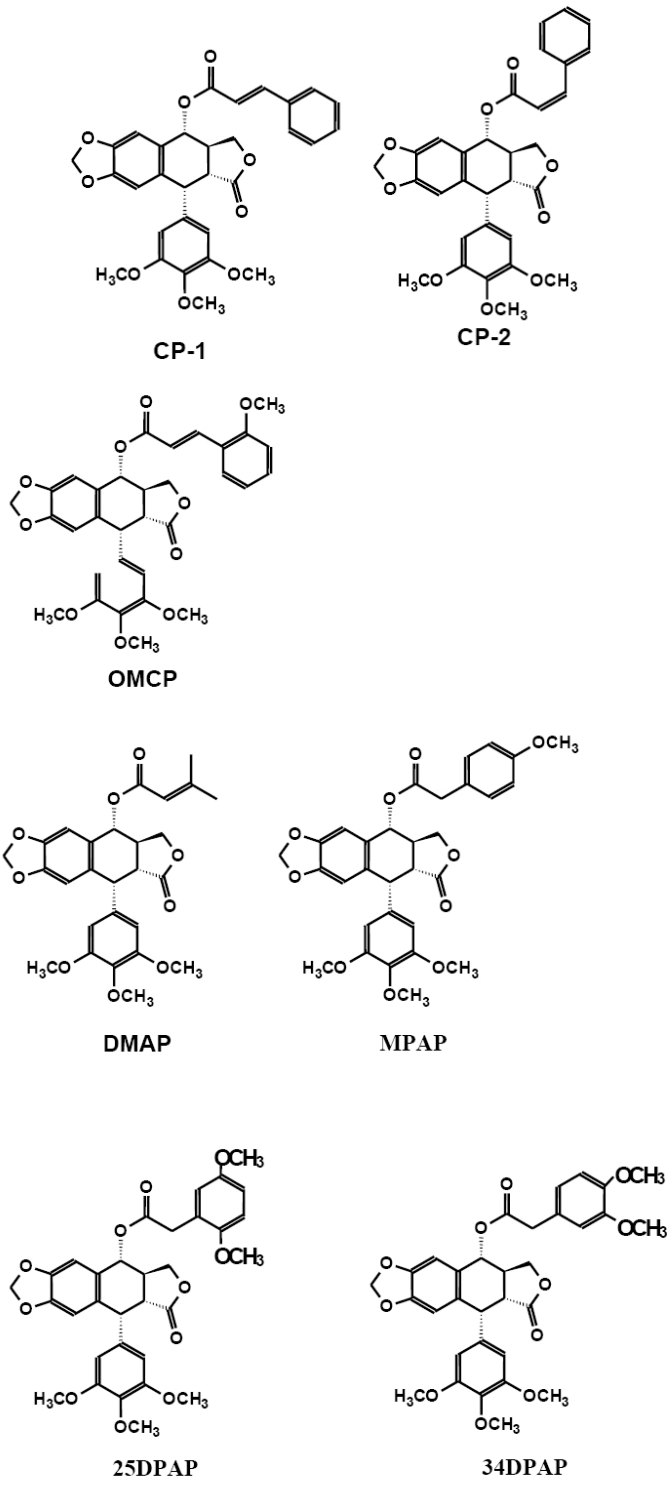

Fig. 2 Structure of podophylotoxin derivatives.

The compounds were characterised and their structures and purity were confirmed using melting point apparatus, ^{1}H-NMR, ^{1}3C NMR, FTIR, and MS. ^{1}H and ^{1}3C NMR spectra were recorded on a Varian Gemini-300 or Inova 500 NMR spectrometer operating

at 400 and 125 MHz, respectively. The chemical shifts were reported as parts per million (ppm) units relative to TMS. $CDCl_3$ was used as the solvent for these measurements. IR spectra were recorded on a Nicolet Avatar 360 FT-IR spectrometer. Only noteworthy IR absorptions (cm^{-1}) are listed. UV - VIS spectra (nm) were recorded on a U-2001 Hitachi model using methanol as the solvent. Mass spectra were recorded on a 5995 HP GC/MS. Only prominent fragments are reported. Melting points were determined in a capillary tube and are uncorrected.

2.1 Esterification: General Procedure:

The esterification was done using dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP). The presence of DMAP makes the combination a powerful agent. In this procedure the acid and alcohol were taken in the reaction vessel and DCC was added in stoichometric quantity dropwise, with vigorous stirring. All the compounds synthesised were homogenous as determined by TLC. The melting points were measured in a paraffin bath and those values are given in the Table 1.

The stereochemistry around the carbinol carbon β of the alcohol remained unchanged, as could be observed from the coupling constant of the proton on C-4. Podophyllotoxin has its H-4 resonating at δ 4.73, as a doublet with a coupling constant of $7 - 9$ Hz. All the derivatives synthesised have shown similar coupling constant, though the signal has shifted as expected down field (esterification of a secondary alcohol causes a down field shift of δ by a value of $1 - 1.2$). The stereochemistry across C3-C4 was therefore retained. The δ values observed for H-4 in the ^{1}H-NMR spectrum, the extra ester carbonyl absorption in the IR spectrum, and the diagnostic signals in their mass spectrum of each of the compounds are presented in the Table 1.

Table 1 Diagnostic spectral detail of the derivatives of podophyllotoxin.

No.	Compound	Melting Point (°C)	Rf (PE:EA, 3: 2)	Yield (%)	IR cm^{-1}.	Diagnostic Spectral Signals ^{1}H-NMR $J = 7 - 9$ Hz	Observed	HREIMS
1.	P	180 – 182	0.21	—		1755.8	4.75	414.1315
2.	CP-1	205 – 207	0.71	13.05		1772, 1706.2	5.90	544.1720
3.	CP-2	201 – 203	0.76	36.62		1779, 1709	6.00	544.1726
4.	OMCP	198 – 200	0.69	36.58		1776, 1703	5.98	574.1846
5.	DMAP	214 – 216	0.68	42.11		1769,1718	5.75	496.1728
6.	MPAP	187 – 189	0.524	60.45		1768, 1740	5.70	-
7.	25DPAP	154 – 156	0.53	66.00		1760,1752	5.89	592.1934
8.	34DPAP	121 – 123	0.52	65.20		1775, 1737	5.85	592.1933

2.2 Characterization of the compounds

2.2.1 Characterization of podophyllotoxin (1)

The compound was isolated as white solid, and was recrystallized from methanol to give long needle like crystals. The compound exhibited a purple colour with methanolic sulphuric acid and was found to be homogenous as determined by TLC ($Rf = 0.21$ in 3 : 2, Light petrol: ethyl acetate). The solid melted at $180 - 182$ °C.

The IR spectra showed hydroxy stretching bands at 3464.4 cm^{-1} and the lactone carbonyl stretch at 1775.8 cm^{-1}. The aromatic ether (C − O) stretch absorption bands were observed at 1239.3, 1127.2, 1040.1 and 997.6 cm^{-1}.

In its 1H-NMR spectra, the expected methylenedioxy signal was observed at δ 5.95 (2H, d, $J = 3 - 4$ Hz), the aromatic 1 H signals of the A ring were observed at δ 6.5 (1H, s, H - 8) and δ 7.1 (1H, s, H - 5). Among the three 3 methoxyl peaks of the E-ring, two were observed at δ 3.79 (6H, s, 2 × OCH_3) and the other at 3.82 (3H, s, OCH_3). The aromatic signals of the E-ring resembled that of pyrogallol showing a 2H signal at δ 6.35 (2'- H, 6' - H). The signal observed at m/z 168 in the mass spectrum of the compound accounts for the trimethoxy phenyl fragment. Successive loss of the methyl group gives the other peaks at m/z 153, 139 and 126.

The H -1 proton was observed at δ 4.1 (1H) and the signals observed at δ 3.22 (1H) and 2.8 (1H, m) could be attributed to the protons H - 2 and H -3 respectively. The H - 4 signal was observed as a doublet at δ 4.73 (1H, d, $J = 7 - 9$ Hz). The coupling constant indicates this to be an axial interaction, indicating the trans nature of the substituents at C3-C4. The protons on Carbon 11 were observed as group of signals in the region $4.45 - 4.62$ (2H).

The molecular ion peak observed at m/z 414 $[M]'^{+}$ accounted for the molecular formula $C_{22}H_{22}O_8$. The fragments obtained due to the loss of hydroxyl (m/z 396), followed by the loss of methyl (m/z 367) and methoxy group (m/z 337) were observed in the Mass Spectra apart from the fragment (m/z 247) obtained after the loss of the trimethoxy phenyl ring. The peak after the loss of the carbon dioxide moiety from the dehydrated fragment was observed at m/z 351.

2.2.2 Characterization CP-1 and CP - 2 as trans and cis cinnamyl podophyllotoxin

The compounds proved to be homogenous as determined by TLC and gave a purple colour on heating after spraying with methanolic sulphuric acid. The HREIMS gave the molecular formula of both the compounds to be $C_{31}H_{28}O_9$. (Observed for $CP - 1 = 544.1720$, and for $CP - 2 = 544.1716$, calculated for $C_{31}H_{28}O_9 = 544.1725$ amu) Apart from the similar stretching and bending absorptions observed in the spectrum of podophyllotoxin, the IR spectra of the compounds $CP - 1$ and $CP - 2$ showed the ester and lactone peaks at 1772.2, 1779.4 and 1706.2, 1709.3 cm^{-1} respectively.

The esterification of secondary hydroxyl group is known to bring about a downfield shift δ $1 - 1.2$ in the methine proton of the secondary hydroxyl. The methine protons, in this case H-4, were observed as doublets at δ 5.90 (1H, d, $J = 7 - 9$ Hz) and δ 6.05

(1H, d, $J = 7 - 9$ Hz) for $CP - 1$ and $CP - 2$ respectively. The values of the coupling constant J, showed that the orientation of the hydroxyl at the C-4 was α as in the original compound, podophyllotoxin. No epimerization was observed. The diagnostic signals for the geometric isomer identification were those observed at δ 6.26 (1H, d, $J = 16$ Hz) and δ 7.69 (1H, d, $J = 16$ Hz) for $CP - 1$, characteristic of trans coupled protons and the corresponding signals at δ 6.50 (1H, d, $J = 12$ Hz) and at δ 7.74 (1H, d , $J = 12$ Hz) for the $CP - 2$, typical of cis protons. The aromatic signals of the substituted acid were observed at δ 7.40 (3H), 7.51 (2H) and 7.42 (3H), 7.54 (2H) for $CP - 1$ and $CP - 2$ respectively.

The mass spectra of both compounds gave the molecular ion peak [M]'$^+$ at m/z 544, accounting for $C_{31}H_{28}O_9$, i.e., the hydroxyl of podophyllotoxin esterfied with a cinnamyl group. Apart from the signals arising out of the fragmentation of podophyllotoxin, there were peaks corresponding to the cinnamic acid side chain at m/z 147 [R−COO]'$^+$, 131[R−CO]'$^+$, and 103 [R]'$^+$. These peaks were particularly prominent in the cis isomer.

Based on the above observation the compound $CP - 1$ was characterized as trans cinnamyl podophyllotoxin and the compound $CP - 2$ was characterized as cis cinnamyl podophyllotoxin with the stereochemistry of the ester group being α at the C- 4 position.

2.2.3 Characterization of $CP - 1$ as trans cinnamyl podophyllotoxin ($C_{31}H_{28}O_9$)

$CP-1$ was obtained as a white solid that was recrystallized form methanol. It was found to be homogenous as determined by TLC.

Mp. : $205 - 207$ °C.

Rf. : 0.708 (3:2, light petrol ($60 - 80$ °C): ethyl acetate)

IR (KBr) $\nu_{max.}$: 1772.2, 1706.2, 1592.3, 1501.7, 1483.3, 1251.5, 1169.0, 1127.9 and 846 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 7.38 (1H, d, $J = 15 - 16$ Hz, cinnamyl proton), 7.47(2H, m, cinnamyl aromatic protons), 7.4 (3H, m, cinnamyl aromatic protons), 6.82 (1H, s, H - 5), 6.58 (1H, s, H - 8), 6.44 (2H, s, 2' , 6' - H), 6.27 (1H, d, $J = 15 - 16$ Hz, cinnamyl proton), 5.94 (2H, d, $J = 4 - 6$ Hz , O - CH_2 - O -), 5.90 (1H, d, $J = 7 - 9$ Hz, H - 4,), $4.3 - 4.45$ (2H, m, H - 11), 3.85 (3H, s, 4' - OMe), 3.80 (6H, s, 3', 4' - OMe), 3.35 (1H, m, H - 3), 3.1 (1H, m, H - 2).

Mass Spectra (DIP) m/z : [M]'$^+$ 544(22.52), 414 (7.27), 396 (100), 367 (12.61), 338 (13.51), 229 (14.41), 168 (67.56), 147 (54.05), 131 (42.34), 103 (44.14) and 91(30.63).

HREIMS [M$^+$] : Observed 544.1720, (calculated for $C_{31}H_{28}O_9$= 544.1733 amu)

2.2.4 Characterization of $CP - 2$ as Cis cinnamyl podophyllotoxin ($C_{31}H_{28}O_9$)

$CP - 2$ was obtained as a white solid recrystallized form methanol. It was found to be homogenous as determined by TLC.

Mp. : $201 - 203$ °C.

Rf. : 0.708 (3:2, light petrol ($60 - 80$ °C): ethyl acetate)

IR (KBr) $\nu_{max.}$: 2930.3, 2851, 1779.4, 1709.3, 1630.7, 1484.2, 1240.5, 1162.9, 1120.3 and 862.8 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 7.74 (1H, d, $J = 12$ Hz, cinnamyl proton), 7.54 (2H, m, aromatic cinnamyl protons), 7.42 (3H, m, aromatic cinnamyl protons), 6.84 (1H, s, H - 5), 6.58 (1H, s, H - 8), 6.5 (1H, d, J = 12 Hz, Cinnamyl proton), 6.42 (2H, s, H - 2', 6'), 6.00 (1H, d, $J = 7 - 9$ Hz, H - 4), 5.98 (2H, d, $J = 4 - 6$ Hz , O - CH_2 - O -), 4.43 (2H, m, H -11), 4.25 (1H, unresolved doublet, H - 1), 3.79 (3H, s, para -O Me), 3.76(6H, s, 2 × meta -O Me), 3.43 (1H, m, H - 2) and 2.97 (1H, m, H - 3)

Mass Spectra (DIP) m/z (%) : 544 (57.65), 396 (57.65), 338 (13.51), 224 (24.32), 185 (56.75), 168 (76.57), 131 (100), 103 (94.31) and 91 (40.00).

HREIMS [M^+] : observed 544.1726 (calculated for $C_{31}H_{28}O_9$ = 544.1733 amu)

2.2.5 Characterization of OMCP ortho methoxy cinnamyl podophyllotoxin

The compound OMCP was obtained as white solid and was recrystallized from methanol. It was homogenous as determined by TLC and gave a purple colour with methanolic sulphuric acid. The molecular formula of the compound was found to be $C_{32}H_{30}O_{10}$ from the HREIMS data.

In its IR spectrum, the compound showed the additional carbonyl absorption at 1776.5 cm^{-1} apart from the absorption at 1703.2 cm^{-1}.

The mass spectrum showed the signals arising from podophyllotoxin and the molecular ion peak was observed at m/z 574, accounting for the methoxy cinnamyl esterified podophyllotoxin. The peaks due to ester side chain were observed at m/z 179 and m/z 161.

The ^{1}H-NMR spectrum of the compound showed the doublet of the esterified methine at δ 5.98 (1H, d, $J = 7-9$ Hz). The other signals arising from the side chain were observed at δ 8.1 (1H, d, $J = 16$ Hz,), 6.48 (1H, d, $J = 16$ Hz) - the trans protons of the cinnamyl group, δ 6.9 (2H) and $7.32 - 7.50$ (2H) - the aromatic signals.

2.2.6 Characterization of OMCP as ortho methoxy cinnamyl podophyllotoxin ($C_{32}H_{30}O_{10}$)

The solid obtained was homogenous as determined by TLC. OMCP was obtained as needle like crystals from methanol

Mp. : $198 - 200$ °C.

Rf. : 0.685 (3:2, light petrol ($60 - 80$ °C): ethyl acetate)

IR (KBr) $\nu_{max.}$: 2930, 2851, 1176.5, 1703.2, 1628.8, 1576.7, 1158.6, 1125.4, 1046.1 and 892.4 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 8.1 (1H, d, $J = 16$ Hz, cimmamyl proton), $7.32 -$ 7.50 (2H, m, cimnnamyl Ar - H), 6.9 (2H, m, cinnamyl Ar - H), 6. 82 (1H, s, H - 5), 6.48 (1H, d, $J = 16$ Hz, cinnamyl proton), 6.42 (1H, s, H - 8), 6.38 (2H, s, H - 2', 6'), 5.98 (1H, d, $J = 7 - 9$ Hz, H - 4), 5.92 (2H, d, $J = 4 - 6$ Hz, - O - CH_2 - O -), $4.40 - 4.58$ (2H, m, H - 11), 4.20 (1H, m, H - 1), 3.82 (3H, s, -OCH_3), 3.78 (6H, s, -OCH3), 3.40 (1H, m, H -2) and 2.94 (1H, m, H -3).

Mass Spectra (DIP) m/z (%) : [M]'$^+$ 574 (0.90), 413 (0.90), 397 (36.03), 385 (2.7), 339 0.90), 313 (0.78), 225 (97.54), 179 (100), 161 (28.82), 140 (1.80) and 100 (4.50).

HREIMS [M^+] : observed = 574.1846 (calculated for $C_{32}H_{30}O_{10}$ = 574.1839)

2.2.7 Characterization of DMAP as dimethyl acrylyl podophyllotoxin

The compound proved to be homogenous as determined by TLC and gave a purple colour with methanolic sulphuric acid. The compound, recrystallized from methanol, melted at $214-216$ °C. The IR spectrum of the compound showed the carbonyl absorptions of both lactone and ester at 1769.3 and 1718.6 cm^{-1}. The other signals were identical to those observed in podophyllotoxin except that the hydroxyl absorption was absent and there were strong geminal dimethyl absorption observed at 1130.6 and 1143.9 cm^{-1}.

The DIP mass spectrum showed the molecular ion peak as expected at $[M]'^{+}$ m/z 496. The dimethyl acrylic acid fragment of the ester side chain was observed at m/z 100 apart from the signals observed for podophyllotoxin.

In its 1H-NMR spectrum, the 4 hydroxyl proton was shifted downfield to δ 5.75 (1H, d, $J = 7-9$ Hz) and the two 3 H singlets of the allyllic groups were observed at δ 1.90 (3H, s) and δ 2.21 (3H, s). The vinylic proton was observed at δ 5.52 (1H, s) as a singlet. The other signals were accounted for by the alchohol, podophyllotoxin. In agreement with the above observations the HREIMS gave the molecular formula to be $C_{27}H_{28}O_9$.

2.2.8 Characterization of DMAP as dimethyl acrylyl podophyllotoxin

The compound DMAP was obtained as a white solid which could be recrystallized form methanol. It was found to be homogenous as determined by TLC.

Mp. : $214-216$ °C.

Rf. : 0.688 (3:2, light petrol ($60-80$ °C): ethyl acetate)

IR (KBr) $\nu_{max.}$: 2935.9, 1769.3, 1718.6, 1590.1, 1143.9, 1130.0, 1083.6, 1042.2 and 886.9 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 6.75 (1H, s, H - 5), 6.5 (1H, s, H - 8), 6.4 (2H, s, H - 2', 6'), 5.99 (2H, d, $J = 4-6$ Hz, - O - CH_2 - O -), 5.75 (1H, d, $J = 7-9$ Hz, H - 4), 5.52 (1H, s, H - vinyllic), $4.42-4.35$ (1H, m, H - 11), 4. 3 (1H, d, $J = 4-6$ Hz, H - 1), 3.85 (6H, s, $2 \times OCH_3$), 3.80 (3H, s, OCH_3), 3.4 (1H, m, H - 3), 3.27 (1H, dd, $J = 8.3$, H - 2), 2.2 (3H, s, trans CH_3) and 1.9 (3H, s, cis CH_3)

Mass Spectra (DIP) m/z (%) : 496 (42.77), 396 (100), 338 (18.18), 313 (10.27), 168 (35.45), 121(10.00), 100 (20.00) and 91 (12.72).

HREIMS [M^+] : observed = 496.1728 (calculated for $C_{27}H_{28}O_9$ = 496.1733)

2.2.9 Characterization of MPAP as para methoxy phenyl acetyl podophyllotoxin

The compound was homogenous as determined by TLC and melted at 187 - 189 °C. The white solid was recrystallized from methanol to give long needle like crystals. The IR spectrum showed the two carbonyl absorptions bands at 1768.6 and 1740.5 cm^{-1}.

The ^{1}H-NMR spectrum of MPAP displayed the H-4 signal at δ 5.70 (1H, d, $J = 7-9$ Hz), illustrating the down field shift of the methine proton on esterification. The benzylic protons of the acid part of the ester were observed at δ 3.46. Apart from the additional 3 H methoxyl signal observed in the region δ $3.85-3.92$, the 1, 4 substitution of the benzene ring in the side chain was illustrated with the typical AB split pattern observed. The remaining signals could be explained as per the discussion on podophyllotoxin. The

mass spectrum did not give the molecular ion but gave the peaks arising from the side chains at m/z 126 apart from the fragments common to podophyllotoxin.

2.2.10 Characterization of MPAP as 4-methoxy phenyl acetyl podophyllotoxin

The solid obtained from the column was recrystallized in methanol. The compound proved homogenous as determined by TLC.

Mp. : 187 − 189 °C.

Rf. : 0.524 (3:2, light petrol (60 − 80 °C): ethyl acetate)

IR (KBr) $\nu_{max.}$: 2930.7, 2851.0, 1768.6, 1740.5, 1626.2, 1513.5, 1244.8, 1130.1, 1035.5 and 848.2 cm^{-1}.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 7.7 (2H, d, $J = 8$ Hz, H - 5", 3"), 6.90 (2H, d, $J = 8$ Hz, H - 2", 6"), 6.68 (1H, s, H - 5), 6.52 (1H, s, H - 8), 6.40 (2H, s, H - 4', 6'), 5.97 (2H, d, $J = 4 - 6$ Hz, - O - CH_2 - O -), 5.70 (1H, d, $J = 7 - 9$ Hz, H - 4), 4.20 − 4.32 (2H, m, H - 11), 4.08 (1H, m, H - 1), 3.92 (3H, s, OCH_3), 3.9 (6H, s, 2 × OCH_3), 3.85 (3H, s, OCH_3), 3.46 (2H, s, -CO - CH_2- Ph), 3.25 (1H, dd, $J = 8.2$, H - 2) and 2.90 (1H, m, H - 3).

Mass Spectra (DIP) m/z (%) : 224 (51.44), 149 (1.78), 143 (62.72), 121 (8.18) and 99 (100).

2.2.11 Characterization of 25DPAP and 34DPAP as 2,5-dimethoxy phenyl acetyl and 3,4-dimethoxyphenyl acetyl podophyllotoxin.

The compounds 25DPAP and 34DPAP were found to be homogenous as determined by TLC. 25DPAP melted at 154 − 156 °C and 34DPAP melted at 121 − 123 °C. Both the compounds were recrystallized from methanol and gave a purple spot with methanolic sulphuric acid. The IR spectra of 25DPAP and 34DPAP showed the additional ester carbonyl absorption bands at 1752.4 and 1737.3 cm^{-1} respectively. Both the compounds showed the expected molecular ion $[M]'^+$ peak at m/z = 592. The fragments due to the dimethoxy phenyl acetyl group were observed at m/z 196 $[RCOO]'^+$, 178 $[RCO]'^+$ and 151 $[R]'^+$ in the DIP mass spectra of the compounds. In the ^{1}H-NMR spectra the H-4 proton had shifted downfield to δ 5.89 (1H, d, $J = 7 - 9$ Hz) and 5.85 (1H, d, $J = 7 - 9$ Hz) for 25DPAP and 34DPAP respectively. The benzyl protons of the acid were observed at 3.7 (2H, s) and δ 3.68 (2H, s) respectively. Two additional 3H singlets were observed in the region 3.8 − 3.9 in both the cases and the aromatic signals were observed at δ 6.77 − 6.80 (3H) and 6.80 − 6.87 (3H). The HREIMS supported the above observations by corresponding to the formula $C_{32}H_{32}O_{11}$ for both the compounds.

The compounds 25DPAP and 34DPAP were therefore characterized to be 2, 5 - dimethoxy phenyl acetyl podophyllotoxin and 3, 4 - dimethoxy phenyl acetyl podophyllotoxin.

2.2.12 Characterization of 34DPAP as 3, 4-dimethoxy phenyl acetyl podophyllotoxin

The solid obtained from the column was recrystallized in methanol. The TLC behaviour of the compound showed it to be homogenous.

Mp. : 121 – 123 °C.
Rf. : 0.515 (3:2, light petrol (60 – 80 °C): ethyl acetate)
IR (KBr) $\nu_{max.}$: 2931.5, 2851.1, 1775.8, 1737.3, 1629.9, 1588.8, 1512.3, 1240.2, 1127.0, 1032.1 and 862.4 cm^{-1}.
^{1}H NMR (200 MHz, $CDCl_3$) : δ 6.87 (3H, s, H - 2", 3", 6"), 6.68 (1H, s, H - 5), 6.50 (1H, s, H - 8), 6.35 (2H, s, H - 2', 6'), 5.95 (2H, d, $J = 4 - 6$ Hz, - O - CH_2 - O -), 5.85 (1H, d, $J = 7 - 9$ Hz, H - 4), 4.58 (1H, d, $J = 4 - 6$ Hz, H - 1), 4.25 – 4.35 (2H, m, H - 11), 3.95 (6H, s, 2 × -OCH_3), 3.87 (3H, s, -OCH_3), 3.78 (6H, s, 2 × -OCH_3), 3.68 (2H, s, -OC - CH_2 - Ph -), 3.45 (1H, m, H - 2) and 2.87 (1H, m, H - 3).
Mass Spectra (DIP) m/z (%) : $[M]'^+$ 592 (8.18), 396 (26.36), 338 (6.36), 313 (12.72), 224 (51.88), 196 (18.18), 178 (40.90), 151 (100), 143 (52.72) and 99 (83.73).
HREIMS [M^+] : observed = 592.1933 (calculated for $C_{32}H_{32}O_{11}$ = 592.1945)

2.2.13 Characterization of 25DPAP as 2, 5-dimethoxy phenyl acetyl podophyllotoxin

2, 5-DPAP was obtained as a white solid that gave needle like crystals from methanol. It proved homogenous as determined by TLC.
Mp. : 154 – 156 °C.
Rf. : 0.53 (3:2, light petrol (60 – 80 °C): ethyl acetate)
IR (KBr) $\nu_{max.}$: 2930.4, 2851.1, 1752.4, 1760, 1237.3, 1154.1, 1125.6 and 1047.5 cm^{-1}.
^{1}H NMR (200 MHz, $CDCl_3$) : δ 6.77 – 6.80 (3H, H - 2", 4" and 5"), 6.73 (1H, s, H - 5), 6.5 (1H, s, H - 8), 6.27 (2H, s, H - 2', 6'), 5.99 (2H, d, $J = 4 - 6$ Hz, - O - CH_2 - O -), 5.89 (1H, d, $J = 7 - 9$ Hz, H - 4), 4.35 – 4.60 (2H, m, H - 11), 4.21 (1H, m, H - 1), 3.85 (3H, s, OCH_3), 3.8 (6H, s, 2 × OCH_3), 3.73 (6H, s, 2 × OCH_3), 3.70 (2H, s, -CO - CH_2 - Ph), 3.44 (1H, m, H - 2) and 2.84 (1H, s, H - 3).
Mass Spectra (DIP) m/z (%) : $[M]'^+$ 592 (77.62), 397 (100), 367 (8.18), 351 (14.54), 313 (54.45), 229 (54.45), 196 (67.27), 185 (97.27), 168 (53.34), 151 (86.63), 137 (70.00), 121 (67.27) and 91 (49.09)
HREIMS [M^+] : observed = 592.1934 (calculated for $C_{32}H_{32}O_{11}$ = 592.1945)

2.3 Anti fungal activity studies

The routine use of a wide variety of fungicides in agriculture has now become an accepted practice. However the use of these chemicals over the years has resulted in the development of resistance in fungal populations. This problem has created avenues for the synthesis and use of newer and effective fungicides, which are capable of giving selective and efficient protection to crops over a long period of time.

In order to study the effect of substituents on the fungitoxic activity of podophyllotoxin, the synthesised ester derivatives of podophyllotoxin were tested against four species of plant pathogenic fungi. They were:

a) *Macrophomina phaseolina* (root rot pathogen) fungi *Imperfecti.* Known to cause the root and stem rot in jute.

b) *Fusarium oxysporum* (root rot pathogen) fungi /*Imperfecti.* Causes the vascular wilt

of cotton.

c) *Myrthecium verrucarri* (root rot pathogen) fungi *Imperfecti*. Effects the seeds of com, beans, tomato and tobacco.

d) *Aspergillus candidus* (leaf spot pathogen) fungi *Ascomycotina*. It is a seed borne fungi which causes mouldy grains and damages the seeds of any plant by producing protolytic enzymes.

The fungitoxic activity of the ester derivatives of podophyllotoxin was measured using the turbidity method. 5 ml fractions of the homogenized solution were taken in 25 ml test tubes and sterilized in an autoclave for a period of 45 min. 5 ml of the sterilised medium was inoculated with a pathogenic fungi and the fungus was incubated at 31 °C for a period of 24 h. To the 24 h culture 0.2 ml of the test solution (0.1 ppm concentration of the compounds in dimethyl formamide (DMF)) was added. The test tubes were shaken well and incubated for 48 h at 31 °C. DMF was used as the control solvent. DMF as a solvent for antifungal studies has been reported in literature [19]. The extent of inhibition of the compound (anti-fungal activity) was determined by measuring the decrease in turbidity in terms of % transmission at 660 nm. Lower turbidity value indicated higher antifungal activity for the compound. Since DMF was used as the control in the spectroscopy, its antifungal effect was nullified. The fungicidal activity of the Podophyllotoxin derivatives were defined as $(= \log(p/100 - p))$, where p is the relative % transmission

2.4 Quantitative structure activity relationship studies

The structure of the various molecules shown in Figures 1 and 2 were drawn and their minimum energy conformation was determined using Cerius^2 software® using the Universal force field (Acceryls Inc, USA). Two hundred and forty nine descriptors that included topological, charge, geometrical, aromaticity indices, constitutive properties, quantum mechanics and thermodynamics were evaluated for each compound. Several literature reports give a very detailed description of these descriptors [20–22]. Equations were developed between the observed activity and the descriptors. The set of descriptors that would give the statistically best models were selected from the large pool using a Genetic function approach. The best model was selected based on the r^2, r^2_{adj}, F-ratio and $q^2 \cdot r^2$ is an indication of the model data fit. The predictive capability of the equation (q^2) is determined using leave-one-out cross validation method. The relation for q2 is as shown below,

$$q^2 = 1 - PRESS/TOTAL$$

$\sum(Y_{predicted} - Y_{observed})^2$ is the predictive error sum of squares (=PRESS) using the equation that considers $(n-1)$ data points. $\sum(Y_{observed} - Y_{mean})^2$ is the total sum of squares (=TOTAL), where $Y_{predicted}, Y_{observed}$, and Y_{mean} are the predicted, observed, and mean values of activity respectively. A large F indicates that the model fit is not a chance occurrence. R^2 and R^2_{adj} above a value of 0.6 indicate good model fit while q^2 above 0.55 indicates good predictive capability for the model.

The problem that is faced frequently by a researcher is that of a small number of observations (experiments) and a large number of molecular parameters in the descriptor pool. One has to select the best set of descriptors that represent the molecule from this large set. At times selecting the wrong set of descriptors could lead to chance correlations or incorrect understanding. According to researchers the quality of a QSAR depends on two factors namely, the kind of molecular descriptors selected and the method used to extract the useful molecular information. These problems are addressed by the use of GFA. This is a useful technique for searching in a large parameter space when the data is small. This technique can be used together with standard regression analysis for constructing QSAR. This method provides multiple models that are created by evolving random initial models using different descriptors. Models are improved by performing a crossover operation to recombine terms of better scoring models. The GFA algorithm approach has a number of important advantages over other techniques such as, it builds multiple models rather than a single model and it automatically selects which features are to be used in the models. GFA has been used by other researchers as well to develop good QSAR models. Generally, a regression model with one descriptor for five data points is the accepted norm [23, 24].

3 Results and Discussion

3.1 Biologic activity of derivates of Podophyllotoxin

The anti fungal activity results are given in Table 2. As can be clearly seen from Table 2, all the ester derivative of podophyllotoxin exhibited high fungicidal activity against *Fusarium oxyspornm* and moderate activity against *Macrophomina phaeseolina.* Ortho methoxy cinnamoyl ester exhibited almost the highest activity against these two fungi. The activities of the esters against *Alyrthecium vemlcarria* and *Aspergillus candidus* were found to be either low or negative.

Table 2 Fungicidal activity of the ester derivatives of podophyllotoxin.

Compound	Pathogenic Fungi / relative % transmission							
	Mp	Activity	*Fo*	Activity	*Mv*	Activity	*Ac*	Activity
Podophyllotoxin	61.76	++	90.24	+++	44.89	+	35.73	+
Ortho methoxy cinnamoyl ester	64.74	++	96.74	+++	44.22	+	20.04	-
Cinnamoyl ester (trans)	65.31	++	88.17	+++	35.04	+	11.61	-
Cinnamoyl ester (cis)	50.01	++	92.37	+++	48.70	+	20.48	-
Para methoxy phenyl acetoyl ester	57.27	++	93.23	+++	12.54	-	21.79	-
3,4 dimethoxy phenyl acetoyl ester	50.25	++	74.5	++	25.59	+	31.17	+
Dimethyl acryloyl ester	60.39	++ 93.03	+++	16.52	-	34.44	+	
2, 5 dimethoxy phenyl acetoyl ester	56.82	++	86.87	+++	10.05	-	35.67	+

Mp = Macrophomina phaseolina, *Fo = Fusarium oxysporum*, *Mv = Myrothecium verrucama*, *Ac = Aspergillus candidus*, Act. = Activity. (Activity representation: Negative -, Low Positive +, Moderate ++, High +++)

3.2 QSAR studies

The cyto-toxicity of compounds in a physiological system can be attributed to various reasons, such as, inhibition of vital enzymes, irreversible binding with metal ions, interfering with auto-immune response, irreversible binding with DNA, interaction with lipid bi-layer, etc. Historically, most of the earlier well-known toxic compounds and anesthetics found to act by aggregating in the lipid bi-layer, thereby bringing about conformational changes of the lipid proteins and inhibiting the transport of various factors necessary for cell growth across the membrane. Therefore, these groups of compounds did not have any specific three - dimensional - structure - activity - relationships, but their cytotoxicity / anesthetic property could be predicted exclusively based on their physiochemical properties such as Partition Coefficient in n-octanol - water system (Ferguson's rule). These compounds were called *structurally non-specific drugs*. Hence, the hydrophobicity or the lipophilicity of these "structurally non - specific molecules" (obtained from the log P values, calculated using standard quantum mechanical expressions) could itself indicate the toxicity of any the given compound. The compounds whose mechanism of biologic activity, was based on their binding to a target site, thereby, bringing about the observed biologic response, were termed *structurally specific drugs*.

Table 3 lists the best QSAR obtained for the four fungi with the corresponding statistics namely, r^2, r^2_{adj}, q^2, F, and PRESS. The models listed are linear regression equations having one to two descriptors. For all the cases the r^2 is between 0.73 to 0.96, indicating good regression data fit. q^2 for the models are between 0.6 to 0.68, indicating that the predictive capability of the models are acceptable. The model listed for *Macrophomina phaseolina* has one descriptor namely, the hydrophilic-lipophylic partition coefficient ($A\log P$) (Table 4). Compounds with lower $A\log P$ (less hydrophobic) would exhibit higher anti fungal activity against this organism. The solvent accessible surface area appears as a descriptor for the other three systems, specifically the partial positive solvent-accessible surface area. The lipophylicitiy of the compound (Foct) appears as a descriptor for *Fusarium oxysporum*, and as per the model, this value will be its anti fungal activity. Highest occupied molecular orbital (HOMO) appears as a descriptor in the case of *Aspergillus candidus*. Molecular energy appears as a descriptor in the case of *Myrthecium verrucarri*. Energy of the molecule is an indication of its thermodynamic stability and is correlated to the heat of formation. The literature reports the use of structural descriptors for developing QSAR for several fungi [14, 17]. The topology descriptor has been used for developing QSAR for predicting the activity of small organic molecules towards *Fusarium Rosium* and *Aspergillus niger* [25]. Structural, thermodynamic and molecular operating environment descriptors have been reported by researchers developing QSAR for podophyllotoxin compounds [5, 10]. LUMO (energy of the lowest unoccupied molecular orbital) as a descriptor in the QSAR equation has been reported for *Aspergillus nidulans* treated with chlorinated aliphatic hydrocarbons [20].

Fig. 3 shows the parity plot relating the model activity predictions with the actual data for all the four fungi. The figure indicates that the models developed fit the data well.

Table 3 QSAR for various fungal activities.

	Fungus	Equation for activity	r^2	r^2_{adj}	q^2	F	PRESS
1	*Fo*	$1.289 - 0.0308$JursPNSA3 $+ 0.0755$Foct	0.75	0.65	0.60	17.8	0.3
2	*Mp*	$0.392 - 0.087A \log P$	0.73	0.69	0.62	16.7	0.02
3	*Mv*	$0.1698 + 0.0667$ Energy -0.00713 JursDPSA1	0.83	0.76	0.68	12	0.34
4	*Ac*	$-10.386 - 0.4059$HOMO$+7.92$JursFPSA1	0.96	0.94	0.63	53.8	0.2

Mp - Macrophomina phaseolina, Fo - Fusarium oxysporum, Mv - Myrthecium verrucarri and *Ac - Aspergillus candidus*

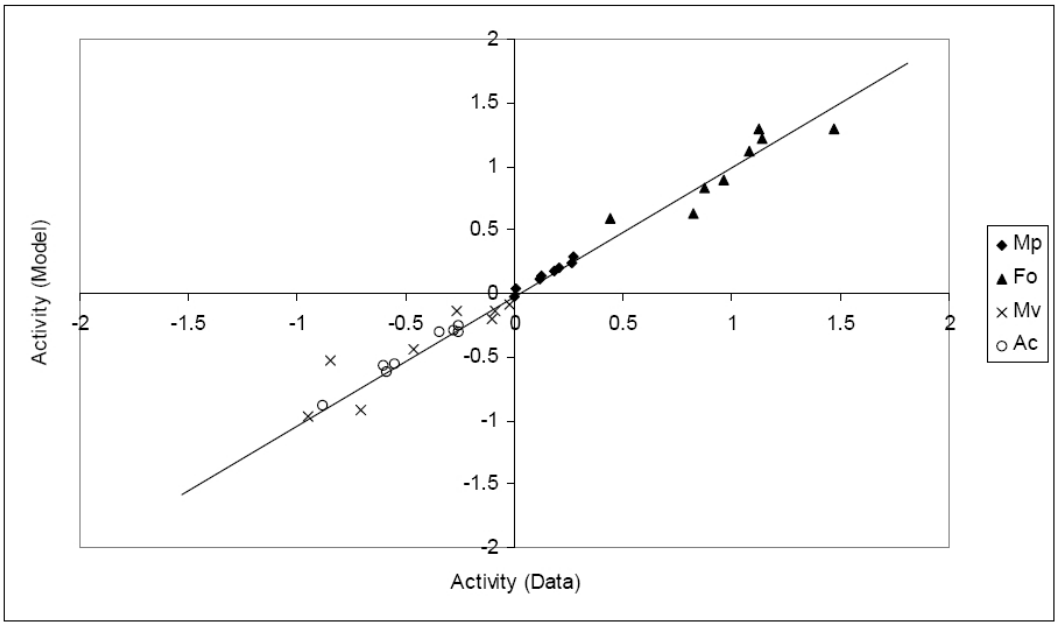

Fig. 3 Parity plot relating model predictions with experimental data against four fungi.

Table 4 Details of descriptors used in the models.

Descriptors	Description
Jurs PNSA3	Atomic charge weighted negative surface area: sum of the product of solvent-accessible surface area x partial charge for all negatively charged atoms
$A \log P$	octanol/water partition coefficient-calculated from group contributions
Foct	1-octanol desolvation free energy, in kcal mol^{-1}.
Energy	The minimum energy of the molecular conformation
Jurs DPSA1	Difference in charged partial surface areas: partial positive solvent-accessible surface area minus partial negative solvent- accessible surface area
HOMO	Highest occupied molecular orbital
Jurs FPSA1	Partial positive surface area: sum of the solvent-accessible surface areas of all positively charged atoms divided by total molecular solvent-accessible surface area

4 Conclusions

The observation that the incorporation of hydrophilic / lipophilic groups on the C-4 hydroxyl group of podophyllotoxin alters its activity, reducing its toxic side effects, was used to our advantage introducing pharmacologically active groups at C-4 OH. The antifungal activities of these synthesised derivatives against four fungi that are pathogenic to plant species were investigated. All the derivatives showed high activity against *Fusarium oxysporom*, moderate activity against *Macrophomina phaseolina* and lowest activity against *Aspergillus candidus*. Through the QSAR studies, it was deduced that the mechanism of action of these derivatives against all the fungi could be akin to that shown by structurally non-specific drugs. Descriptors pertaining to solvent accessible area, octonal water partition coefficient appear in all the QSAR. One and two parameter models appeared to be sufficient to fit the experimental data and the models have reasonably good predictive capability ($q^2 > 0.6$). This study paves the way for the development of ligand based effective anti-fungal agents, while providing an insight into the mode of action of these types of compounds.

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