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Synthesis and cytotoxicity of epoxide and pyrazole analogs of the combretastatins

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Abstract—Twenty-six epoxide and corresponding pyrazole derivatives, of the structurally related chalcones and combretastatin A-4 (**CA-4**), were synthesized and tested for in vitro cytotoxicity. These molecules were synthesized by epoxidation of the relevant chalcones, followed by reaction with hydrazine. The structures of epoxides **3** and **7**, and pyrazole **17**, were confirmed by X-ray diffraction studies. The relatively coplanar conformation of a 3′,3″,4′,4″,5′,5″-hexamethoxypyrazole **17** was in good agreement with the shape for 3′,3″,4′,4″,5′-pentamethoxypyrazole **16**, which was determined from molecular mechanics optimization. In vitro cytotoxicity of each class of compounds was obtained using a 72 h continuous exposure MTT assay against two murine cancer cell lines; B16 melanoma and L1210 leukemia. The effect of substitution in the A-ring is addressed: three methoxy groups versus two, generally increased cytotoxicity across both cell lines. In the majority of cases, the pyrazoles are generally more active than the epoxides, with the most active, 5-(3″-amino-4″-methoxyphenyl)-3-(3′,4′,5′-trimethoxyphenyl)pyrazole **21**, possessing an IC_{50} value of 5 and 2.4 μM (B16 and L1210, respectively). Due to their planar conformations, the pyrazoles are typically less active than the corresponding chalcones, which adopt angular conformations similar to **CA-4**. B-ring modifications confirmed that in general the amino compounds are more active than the corresponding nitro compounds. Varying the number and orientation of methoxy groups on the A-ring did not produce any significant differences in toxicity in the cell lines studied.

1. Introduction

Research into the antitumor properties of chalcones has received significant attention over the last few years, particularly with the discovery that these compounds possess a similar mode of action to the structurally related combretastatins.[1] Combretastatin A-4 (**CA-4**, Fig. 1) is isolated from the African willow tree (*Combretum caffrum*)[1b] and binds to the colchicine site of tubulin, thus preventing tubulin polymerization.[2–5] Furthermore, **CA-4** interrupts metaphase of the cell cycle: the lack of microtubules (primarily composed of tubulin) prevent the formation of mitotic spindles.[6] Moreover, tumor growth is also delayed and inhibited by the obstruction of blood flow in tumor microvasculature.[7]

The development of **CA-4** as a potential antitumor drug has been compromised by its poor solubility in biological media and low bioavailability. This physiochemical limitation led to the development of a water soluble phosphate derivative (**CA-4P**, Fig. 1), designed to improve in vivo efficacy. This prodrug is currently undergoing clinical evaluation for the treatment of cancer.[8] Another derivative of **CA-4** that shows excellent potency and improved solubility in biological media is **AC-7739** (Fig. 1), which has an amino group in place of the hydroxyl moiety in **CA-4**.[3] The aforementioned limitations of the combretastatins have fuelled further research into structural analogs of these compounds with the goal of enhancing bioavailability and antitumor activity. A wide range of structural analogs have been reported, which include substitution of the A- and/or B-ring in the combretastatin frame-

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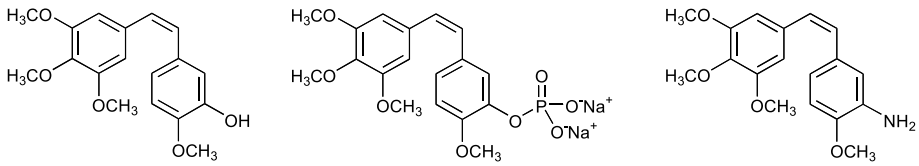


Figure 1. Structure of combretastatin A-4 (**CA-4**), the water soluble prodrug (**CA-4P**), and an amino derivative (**AC-7739**).

work with different heterocycles.[9] B-ring modifications have also included aroylindoles,[10] quinoline, and quinoxaline moieties.[11] Replacing the stilbene core with different functionality has also been attempted by several groups, including our own laboratory, for example, enones (the chalcones).[12] We have recently described the synthesis and in vitro cytotoxicity of methoxy-substituted chalcones, comparing the effect of nitro and amino groups on both the A- and B-ring.[12] Other functional groups that bridge the two aryl moieties have also been investigated, and they include carbonyl[10] and different heterocycles, including, for example, furanones,[13] isoxazoles,[14] imidazoles,[15] triazoles[16], and azetidinone[17] compounds; all of which possess varying levels of cytotoxicity. The rationale behind the central heterocyclic ring is to 'lock' the molecule into the *cis*-orientation; identified previously as crucial for activity.

During our design of novel derivatives of chalcones, as analogs of the combretastatins, with tubulin binding and antitumor properties, it was apparent that heterocyclic derivatives of chalcones had not been systematically investigated for this purpose. Eleven chalcone epoxide precursors (**1**–**11**), and the fifteen 3,5-diarylpyrazoles (**12**–**26**), were synthesized and studied to establish the cytotoxicity. These compounds were designed to test any structure–activity relationships arising from the number and position of methoxy groups in the A- and B-rings, and the effect of a nitro versus an amino group on cytotoxicity. Both classes of compounds were assessed for biological activity against the growth of two murine cell lines (B16 and L1210) grown in culture. Several 3,5-diarylpyrazoles have been prepared and studied as ligands in transition metal chemistry[18] and some have been proposed to have herbicidal properties.[19] Substituted 3,5-diarylpyrazole compounds are particularly intersting as they can be readily prepared from their corresponding chalcones, via the corresponding chalcone epoxide, di- or α-bromochalcones.[19,20] They can also be synthesized by 1,3-dipolar cycloadditions of diazo compounds generated in situ.[21]

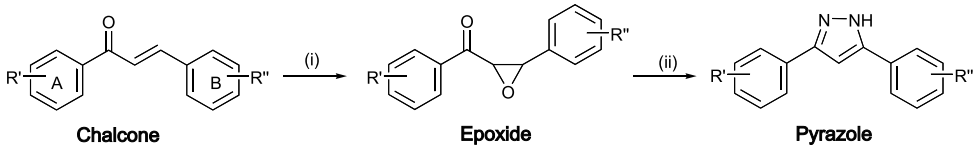
2. Results and discussion

2.1. Synthesis

Synthesis of the chalcones was achieved in high chemical yields using the Claisen–Schmidt condensation of functionalized acetophenones and benzaldehydes using the published method.[12] The general method used in the synthesis of the epoxides and the subsequent pyrazoles is depicted in Scheme 1. Chalcones were treated with powdered potassium carbonate in methanol followed by excess hydrogen peroxide. The epoxides (**1**–**11**, Table 1) were isolated as pure solids (in most cases) in good yields (67–89%). The functionalized pyrazoles were synthesized by dissolving the epoxide in xylenes and treating with *p*-toluenesulfonic acid and hydrazine hydrate and the solution stirred at reflux until the product precipitated (**12**–**18**, **20**, **22**, **23**, **25**, Table 1). Removal of the xylenes and washing with hexane produced the pure products in good yields (43–95%). The pyrazole synthesis, via the epoxide intermediate, allows relatively large numbers of compounds to be produced readily without the need for tedious purification, as the crude product in most cases, is sufficiently pure and can be used directly. The synthetic strategy reported in this paper improves on yield, reaction time, and purification compared to reported approaches.[13,19]

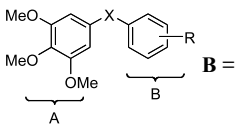
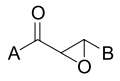
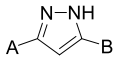
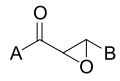
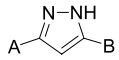
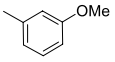
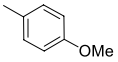
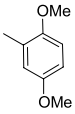
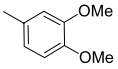
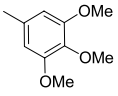
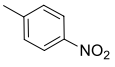
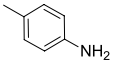
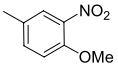
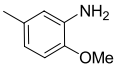
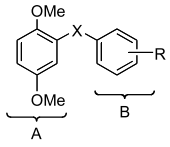
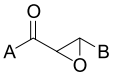
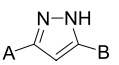
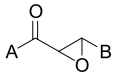
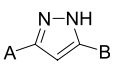
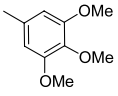
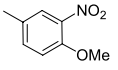
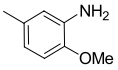
Several attempts were made to prepare the amino epoxide compounds by reduction of the nitro precursors (**10**, **11**, **23**, **25**) with stannous chloride dihydrate (Scheme 2). In all cases, the amino products were unstable and decomposed during the work-up procedures. The aminopyrazoles (**19**, **21**, **24**, **26**) were prepared in 78%–95% yields by atmospheric pressure catalytic hydrogenation over 5% palladium-on-carbon (Scheme 2). The structures of all compounds described in this study were characterized using FT-IR, 500 MHz ^{1}H NMR, mass spectrometry, and accurate mass measurements.

Crystals of epoxides **3** and **7**, and pyrazole **17** were obtained following recrystallization from hexane/ethyl acetate and their structures were unequivocally confirmed

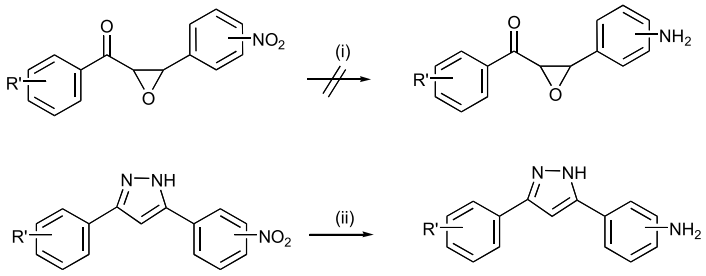


Scheme 1. Synthesis of epoxides and pyrazoles from the starting chalcones. Reagents and conditions: (i) K_2CO_3, MeOH, room temperature, H_2O_2 (ii) Hydrazine hydrate, *p*-toluenesulfonic acid, xylenes, Δ.

Table 1. Cytotoxicity data for compounds **1**–**26**

	IC_{50} (μM)			
	B16		L1210	
	X = 	X = 	X = 	X = 
	>100 (**1**)	55 (**12**)	40 (**1**)	15 (**12**)
	25 (**2**)	38 (**13**)	5 (**2**)	24 (**13**)
	>100 (**3**)	46 (**14**)	>100 (**3**)	30 (**14**)
	>100 (**4**)	40 (**15**)	45 (**4**)	40 (**15**)
	>100 (**5**)	35 (**16**)	81 (**5**)	24 (**16**)
	38 (**6**)	34 (**17**)	32 (**6**)	24 (**17**)
	29 (**7**)	>100 (**18**)	3.9 (**7**)	>100 (**18**)
	N/A[a]	>100 (**19**)	N/A[a]	43 (**19**)
	31 (**8**)	32 (**20**)	5.3 (**8**)	37 (**20**)
	N/A[a]	5 (**21**)	N/A[a]	2.4 (**21**)
	X = 	X = 	X = 	X = 
	59 (**9**)	50 (**22**)	>100 (**9**)	22 (**22**)
	>100 (**10**)	>100 (**23**)	>100 (**10**)	>100 (**23**)
	N/A[a]	40 (**24**)	N/A[a]	35 (**24**)
	>100 (**11**)	>100 (**25**)	>100 (**14**)	>100 (**25**)
	N/A[a]	>100 (**26**)	N/A[a]	>100 (**26**)

[a] Compounds not tested as the epoxide–amine could not be synthesized due to decomposition of the amine before isolation.



Scheme 2. Synthesis of amino compounds. Reagents: (i) $SnCl_2{\cdot}2H_2O$, EtOAc, Δ. (ii) H_2, 5% Pd/C, THF.

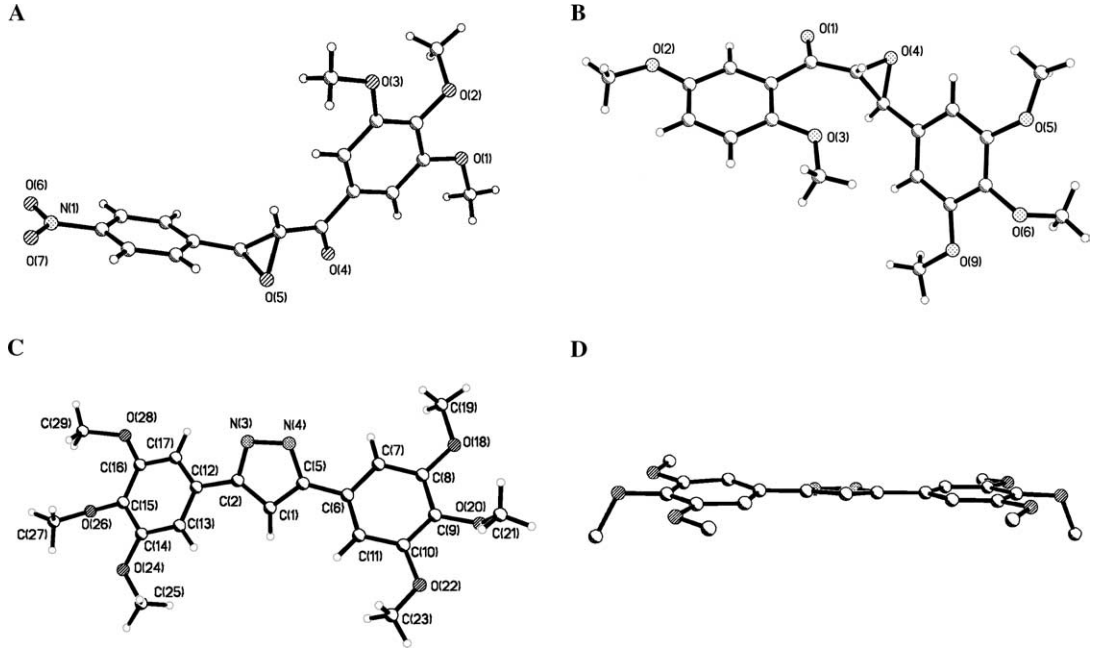


Figure 2. X-ray structures of epoxides, **7** (A) and **3** (B), and pyrazole **17**, aerial view (C) and side view (D).

by single crystal X-ray analysis (Fig. 2). The X-ray structure of compound **17** affirmed the high degree of coplanarity between the aromatic rings. This conformation of pyrazole **17** is in agreement with an MM2 (Cache 3.9) optimized structure of pyrazole **16** (Fig. 3), which differ by one methoxy group (five cf. six). The coplanar con-

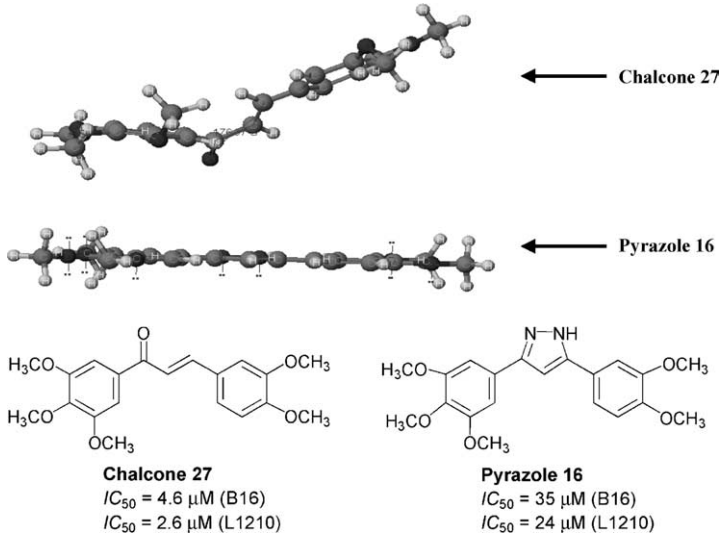


Figure 3. MM2 structures of chalcone **27** and pyrazole **16**. The calculations were performed using CAChe 3.9 software and augmented MM2 parameters. Optimization of each structure was accomplished using a conjugate gradient procedure and a convergence to 0.001 kcal mol^{-1}. The cytotoxicity IC_{50} values for both compounds with B16 and L1210 cells are given in the figure.

formation of compounds **16** and **17** is dramatically different from the angular shape of **CA-4**.[22] Interestingly, the MM2 optimized structure of the pentamethoxychalcone **27**, which exhibits appreciable cytotoxicity, also adopts an angular conformation (Fig. 3), similar to **CA-4**. The difference in conformation between the planar pyrazole compounds **16** and **17** from angular **CA-4** and chalcone **27** could influence their ability to bind to tubulin and inhibit cell growth, assuming the former compounds to have a similar mechanism of action as the chalcones.[23]

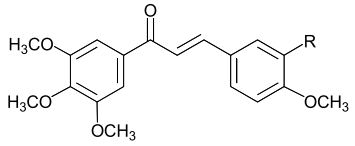
2.2. Cytotoxicity

The epoxides and pyrazoles were subjected to cytotoxicity testing to determine if a structure–activity relationship could be identified. In vitro cytotoxicity experiments were performed on all synthesized compounds using a 72 h continuous exposure MTT assay[24] with murine melanoma and leukemia cells (B16 and L1210, respectively). Ninety-six-well plates were treated with the compound of interest dissolved firstly in DMSO, at concentrations of 1.75×10^{-2} M, then diluted 1 in 10 with media to final concentrations of 1.0×10^{-4}–1.0×10^{-12} M. Concentrations of compounds that inhibited the growth of tumor cells by 50% relative to an untreated control, or IC_{50} (μM) values, for both cell lines are shown in Table 1 (compounds **1**–**26**). The results show that for both the pyrazoles and the epoxides (in both cell lines) the compounds containing three methoxy groups in the A-ring are generally more active than those containing two methoxy groups [e.g., epoxide **6** and pyrazole **16** vs epoxide **9** and pyrazole **21** (Table 1)]. This discovery is consistent with the activity of the combretastatins, such as **CA-4** (IC_{50} = 7 μM in L1210 cells),[25] which also has three similarly positioned methoxy substituents in the A-ring.[26] Comparison of the cytotoxicity of **CA-4** with the L1210 data shown in Table 1 indicates that several of the epoxides are more active in these cells; namely compounds **2**, **7**, and **8**. From the pyrazole series compound **21** is the most active with an IC_{50} of 2.4 μM. The data highlights that both series of compounds are worthy of further investigation.

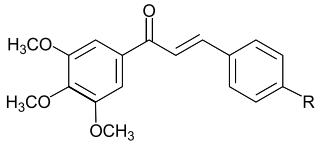
Amino chalcones are in general more active than the corresponding pyrazoles.[12] For example, the IC_{50} values of pyrazole **16** for B16 and L1210 cancer cells were 35 and 24 μM, respectively. In contrast, the cytotoxicity of corresponding chalone **27** (Fig. 3) against the same cancer cells was 4.6 and 2.6 μM, respectively.[12] Moreover, if B16 cells were considered, nitro-chalcone **28** (Fig. 4) shows less activity than the corresponding nitro-pyrazole **20** (cf. > 100[12]–32 μM, respectively). However, the amino-chalcone **29** (Fig. 4) is highly cytotoxic and it has an IC_{50} of 0.24 μM[12] in B16 cells, compared to lower activity for amino pyrazole **21** (IC_{50} of 5 μM). A similar relationship can be observed if chalcones **30** and **31** are considered (Fig. 4), when compared with their respective pyrazoles **18** and **19** (Table 1). This difference in cytotoxicity could be related to their conformation; the pyrazole being coplanar and the chalcones twisted. Guided by the shape of **CA-4** and its conformational relationship to colchicine,[27] it is reasonable to suggest that the chalcones are more cytotoxic than the pyrazoles because they prefer a twisted shape, which allows them to fit more snugly into the binding site of tubulin.[23] These results indicate that shape should be an important factor in the design of future molecules. Despite their angular conformation, and in contrast to the relevant chalcones, the epoxides were less cytotoxic than their corresponding pyrazoles. For example, epoxide **5** was 3–4 times less active than pyrazole **16**, which in turn was 5–15 times less active than the relevant chalcone.

When an amino group is present in the *meta* position of the B-ring, increased activity is seen, in both cell lines, compared with an amine in the *para* position for the trimethoxy compounds [e.g., IC_{50} = >100 and 43 vs 5 and 2.4 μM for pyrazoles **19** and **21** in B16 and L1210, respectively (Table 1)]. The same trend can be observed for the dimethoxy compounds [e.g., IC_{50} = >100 vs 40 and 35 μM for pyrazoles **26** and **24** in B16 and L1210, respectively (Table 1)]. This finding correlates with the potency of **CA-4** which has a hydroxy group in the *meta* position[25] and **AC-7739** that also has an amino group in the *meta* position.[3,28]

Consistent with the chalcone series,[12] increased cytotoxicity is observed when the nitro groups of the epoxides and pyrazoles are reduced to the amines. However, when the amine is present in the *para* position no change in activity is observed for the 2′,5′-dimethoxy compounds (IC_{50} = >100 μM for both **25** and **26** in both cell lines, Table 1). When the 3′,4′,5′-trimethoxy compounds are considered, no change in activity is seen in the B16 cell line (IC_{50} = >100 μM for **18** and **19**, Table 1), however, in the L1210 cell line the activity of the amine compound **19** is increased compared to the nitro, **18** (IC_{50} = 43 and 100 μM, respectively). In general the L1210 cell line appears more sensitive to these two classes of compounds when compared with the B16 results. This was particularly evident within the epoxides, for example, an epoxide **2** with a 3″-methoxy group



28, R = NO_2 (IC_{50} = > 100 μM)
29, R = NH_2 (IC_{50} = 0.24 μM)



30, R = NO_2 (IC_{50} = > 100 μM)
31, R = NH_2 (IC_{50} = 30 μM)

Figure 4. Structures and cytotoxicity of representative chalcones, **28**–**31**. The respective IC_{50} values for B16 cells are given inside the parentheses.

produced IC_{50} values of 25 and 5 μM for B16 and L1210. This difference in activity between cell lines could be due to the nature of the cells. In general leukemia cells (e.g., L1210) are more sensitive to antiproliferative agents than solid tumor cells (B16) which are known to be chemoresistant, shown in the literature with other anticancer agents, particularly the duocarmycins.[29]

The significant enhancement in the activity of amino compounds over their respective nitro counterparts could be useful in the quest for bioreductively active prodrugs. The nitro group could be reduced in vivo to the amino compound, reducing problems with stability and potential nonselective toxicity. For example, **CB1954**, a nitro-compound substrate for DT-diaphorase is reduced in vivo and is active against the growth of cancer cells.[30] Similarly, the nitro moiety of a 4-nitrobenzyloxycarbonyl prodrug of *seco*-cyclopropylindoline (CI) of CC-1065 was effectively reduced by nitroreductase, thereby releasing the active drug.[31]

3. Experimental

3.1. Synthesis

Solvents and organic reagents were purchased from Aldrich or Fisher, and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument and are uncorrected. Infrared (IR) spectra were recorded using a Perkin-Elmer Paragon 500 FT-IR instrument as films on KBr discs, unless otherwise stated. ^{1}H NMR spectra were obtained using a Varian Unity Inova 500 instrument unless otherwise stated. Chemical shifts (δ) are reported at 20 °C in parts per million (ppm) downfield from internal tetramethylsilane (Me_4Si). High-resolution mass spectra (HRMS) and low-resolution mass spectra (LRMS) were provided by the Mass Spectrometry Laboratory, University of South Carolina, Columbia. Reaction progress was assessed by thin-layer chromatography (TLC) using Merck silica gel (60 F_{254}) on aluminium plates unless otherwise stated. Visualisation was achieved with UV light at 254 nm and/or 366 nm, I_2 vapor staining, and ninhydrin spray.

3.2. General preparation of substituted chalcone epoxides (1–11)

Powdered K_2CO_3 (332 mg, 3 mol equiv) was added to a suspension of the required chalcone (300 mg, 1 mol equiv) in MeOH (30 cm^3), followed by excess aqueous hydrogen peroxide (35%, 1 cm^3, 10 mol equiv); added over 10 min. The mixture was stirred at room temperature for 3 h and reaction progress was monitored by TLC (30:70 v/v EtOAc/hexanes). Upon completion, the MeOH was removed under reduced pressure and the resulting residue dissolved in CH_2Cl_2 (50 cm^3), and washed with H_2O (20 cm^3). The organic phase was separated, dried (Na_2SO_4) and the solvent removed under reduced pressure to yield the corresponding epoxide. The compounds were generally pure by TLC and 500 MHz ^{1}H NMR analyses. If required, the compounds were purified by silica gel column chromatography using a 10%–30% gradient ethyl acetate/petroleum ether solvent.

3.2.1. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(2″-methoxyphenyl)propanone (1). White solid (210 mg, 67%), mp 124–126 °C: ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H), 3.90 (s, 6H), 3.93 (s, 3H), 4.11 (d, 1H, J = 1.95 Hz), 4.40 (d, 1H, J = 1.95 Hz), 6.90–6.93 (m, 1H), 6.98–7.04 (m, 1H), 7.29–7.32 (m, 2H), 7.34 (s, 2H); IR (KBr) ν 2936, 2833, 1679, 1581, 1498, 1456, 1415, 1342, 1249, 1160, 1124, 1020, 1000, 756 cm^{-1}; MS (EI) m/z (rel intensity) 344 (M^+, 36). 195 (100).

3.2.2. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(3″-methoxyphenyl)propanone (2). White solid (280 mg, 89%), mp 79–81 °C: ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H), 3.89 (s, 3H), 3.93 (s, 3H), 4.08 (d, 1H, J = 1.8 Hz), 4.18 (d, 1H, J = 1.8 Hz), 6.86–6.95 (m, 3H), 7.25 (s, 2H), 7.26–7.29 (m, 1H); IR (KBr) ν 3620, 3536, 2939, 2834, 1678, 1583, 1494, 1458, 1416, 1337, 1227, 1164, 1128, 1038, 1002, 885 cm^{-1}; MS (EI) m/z (rel intensity) 344 (M^+, 50), 195 (100).

3.2.3. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(4″-methoxyphenyl)propanone (3). Clear oil (170 mg, 54%): ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H), 3.88 (s, 6H), 3.92 (s, 3H), 4.04 (d, 1H, J = 1.5 Hz), 4.20 (d, 1H, J = 1.5 Hz), 7.00 (d, 2H, J = 9.0 Hz), 7.30 (s, 2H), 7.83 (d, 2H, J = 9.0 Hz); IR (KBr) ν 3007, 2941, 2838, 1714, 1678, 1585, 1510, 1461, 1415, 1333, 1252, 1168, 1126, 1029, 1001, 832, 760 cm^{-1}; MS (EI) m/z (rel intensity) 344 (M^+, 55), 195 (100).

3.2.4. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(2″,5″-dimethoxyphenyl)propanone (4). White crystalline solid (516 mg, 82%), mp 81 °C: ^{1}H NMR ($CDCl_3$) δ 3.79 (s, 3H), 3.80 (s, 3H), 3.89 (s, 3H), 3.93 (s, 3H), 4.08 (d, 1H, J = 2.0 Hz), 4.38 (d, 1H, J = 2.0 Hz), 6.85 (s, 1H), 6.86 (s, 1H), 6.88 (s, 1H), 7.26 (s, 1H), 7.33 (s, 1H); IR (KBr) ν 2999, 2936, 2833, 1679, 1586, 1503, 1461, 1415, 1337, 1269, 1217, 1160, 1124, 1041, 1020, 880, 808, 735 cm^{-1}; MS (EI) m/z (rel intensity) 374 (M^+, 46), 195 (100).

3.2.5. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(3″,4″-dimethoxyphenyl)propanone (5). Yellow oil (461 mg, 74%): ^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H), 3.94 (s, 3H), 3.96 (s, 3H), 4.04 (d, 1H, J = 1.95 Hz), 4.17 (d, 1H, J = 1.95 Hz), 6.83–6.99 (m, 2H), 7.25 (s, 2H), 7.40–7.47 (m, 1H); IR (KBr) ν 2999, 2936, 2833, 1679, 1586, 1513, 1461, 1415, 1332, 1269, 1238, 1124, 1020, 859, 808, 756 cm^{-1}; MS (EI) m/z (rel intensity) 374 (M^+, 42), 195 (100); HRMS [M^+] for $C_{20}H_{27}O_7$: obsd 374.1536, calcd 374.1566.

3.2.6. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)propanone (6). Purified by flash column chromatography using silica (30:70 v/v EtOAc–petroleum ether). Yellow solid (416 mg, 80%), mp 83 °C: ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H), 3.84 (s, 6H), 3.86 (s, 3H), 3.90 (s, 3H), 4.02 (d, 1H, J = 1.5 Hz), 4.12 (d, 1H, J = 1.5 Hz), 6.57 (s, 4H); IR

(KBr) ν 3005, 2941, 2837, 1681, 1588, 1504, 1462, 1416, 1327, 1236, 1165, 1127, 1003, 884, 855, 755 cm^{-1}; MS (EI) *m/z* (rel intensity) 404 (M^+, 63), 195 (100); HRMS [M^+] for $C_{21}H_{24}O_8$: obsd 404.1469, calcd 404.1471.

3.2.7. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(4″-nitrophenyl)propanone (7). Yellow solid (462 mg, 78%), mp 140 °C: ^1H NMR ($CDCl_3$) δ 3.91 (s, 6H), 3.95 (s, 3H), 4.18 (d, 1H, J = 1.75 Hz), 4.23 (d, 1H, J = 1.75 Hz), 7.30 (s, 2H), 7.56 (d, 2H, J = 8.25 Hz), 8.28 (d, 2H, J = 8.25 Hz); IR (KBr) ν 3397, 2936, 2843, 1679, 1583, 1520, 1456, 1415, 1342, 1162, 1126, 994, 849 cm^{-1}; MS (EI) *m/z* (rel intensity) 359 (M^+, 55), 195 (100); HRMS [M^+] for $C_{18}H_{17}NO_7$: obsd 359.1010, calcd 359.1005.

3.2.8. 2,3-Epoxy-1-(3′,4′,5′-trimethoxyphenyl)-3-(4″-methoxy-3″-nitrophenyl)propanone (8). Yellow solid (0.23 g, 74%), mp 148 °C: ^1H NMR ($CDCl_3$) δ 3.90 (s, 6H), 3.94 (s, 3H), 3.99 (s, 3H), 4.10 (d, 1H,J = 1.80 Hz), 4.17 (d, 1H, J = 1.80 Hz), 7.13 (d, 1H, J = 8.7 Hz), 7.26 (s, 2H), 7.56 (dd, 1H, J = 8.7 Hz, 2.4 Hz), 7.85 (d, 1H, J = 2.4 Hz); IR (KBr) ν 2999, 2943, 2840, 1679, 1622, 1582, 1534, 1503, 1459, 1416, 1346, 1278, 1163, 1126, 1005, 882, 818, 756 cm^{-1}; MS (EI) *m/z* (rel intensity) 389 (M^+, 35), 195 (100); HRMS [M^+] for $C_{19}H_{19}NO_8$: obsd 389.1106, calcd 389.1111.

3.2.9. 2,3-Epoxy-1-(2′,5′-dimethoxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)propanone (9). White solid (250 mg, 78%), mp 130–132 °C: ^1H NMR ($CDCl_3$) δ 3.61 (s, 3H), 3.81 (s, 3H), 3.86 (s, 3H), 3.88 (s, 6H), 3.96 (d, 1H, J = 1.8 Hz), 4.28 (d, 1H, J = 1.8 Hz), 6.61 (s, 2H), 6.88 (d, 1H, J = 9.0 Hz), 7.09 (dd, 1H, J = 9.0, 3.3 Hz), 7.35 (d, 1H, J = 3.3 Hz); IR (KBr) ν 2995, 2941, 2835, 1663, 1589, 1491, 1454, 1418, 1276, 1265, 1230, 1221, 1189, 1168, 1121, 1022, 1005, 978, 912, 882, 819, 725 cm^{-1}; MS (EI) *m/z* (rel intensity) 374 (M^+, 45), 165 (100).

3.2.10. 2,3-Epoxy-1-(2′,5′-dimethoxyphenyl)-3-(4″-methoxy-3″-nitrophenyl)propanone (10). White-yellow powder (177 mg, 67%), mp 179 °C: ^1H NMR ($CDCl_3$) δ 3.65 (s, 3H), 3.82 (s, 3H), 3.99 (s, 3H), 4.01 (d, 1H, J = 1.8 Hz), 4.32 (d, 1H,J = 1.8 Hz), 6.09 (d, 1H, J = 9.0 Hz), 7.11 (dd, 1H, J = 9.0 Hz, 3.3 Hz), 7.13 (d, 1H, J = 8.8 Hz), 7.35 (d, 1H, J = 3.3 Hz), 7.57 (dd, 1H, J = 8.8 Hz, 2.3 Hz), 7.88 (d, 1H, J = 2.3 Hz); IR (KBr) ν 3061, 2939, 2833, 1672, 1609, 1577, 1541, 1498, 1452, 1418, 1353, 1268, 1226, 1168, 1041, 1018, 888, 836, 730 cm^{-1}; MS (EI) *m/z* (rel intensity) 359 (M^+, 27). 165 (100); HRMS [M^+] for $C_{18}H_{17}NO_7$: obsd 359.1008, calcd 359.1005.

3.2.11. 2,3-Epoxy-1-(2′,5′-dimethoxyphenyl)-3-(4″-nitrophenyl)propanone (11). White-yellow powder (735 mg, 87%), mp 152 °C: ^1H NMR ($CDCl_3$) δ 3.60 (s, 3H), 3.82 (s, 3H), 4.11 (d, 1H, J = 1.8 Hz), 4.31 (d, 1H, J = 1.8 Hz), 6.89 (d, 1H, J = 9.0 Hz), 7.13 (dd, 1H, J = 9.0 Hz, 3.1 Hz), 7.36 (d, 1H, J = 3.1 Hz), 7.56 (d, 2H, J = 8.7 Hz), 8.27 (d, 2H, J = 8.7 Hz); IR (KBr) ν 3082, 2936, 2833, 1677, 1604, 1517, 1496, 1463, 1416, 1346, 1278, 1224, 1169, 1044, 893, 840, 804, 729 cm^{-1}; MS (EI) *m/z* (rel intensity) 329 (M^+, 43), 165 (100).

3.3. General preparation of 3,5-diarylpyrazoles (12–18, 20, 22, 23, 25)

The required epoxide (170 mg, 1 mol equiv) was dissolved in xylenes (4 cm^3). CH_2Cl_2 (2 cm^3) was added, if required, to achieve dissolution. *p*-Toluenesulfonic acid monohydrate (22 mg) and hydrazine hydrate (0.23 cm^3, 3 mol equiv) were then added to the epoxide solution. The reaction mixture was stirred under refluxing conditions for 3 h until a yellow precipitate formed. The xylenes were removed under reduced pressure and the obtained solid washed with hexanes and air dried for 12–14 h to yield the corresponding pyrazole compound. Analyses by TLC and 500 MHz ^1H NMR confirmed their purity. However, in a few cases the products were purified by flash column chromatography using silica gel (2:98 v/v MeOH–$CHCl_3$).

3.3.1. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(2″-methoxyphenyl)pyrazole (12). Yellow solid (131 mg, 63%), mp 78–81 °C: ^1H NMR ($CDCl_3$) δ 3.89 (s, 3H), 3.95 (s, 6H), 4.01 (s, 3H), 6.90 (s, 1H), 7.03–7.10 (m, 2H), 7.11 (s, 2H), 7.31–7.37 (m, 1H), 7.74–7.77 (m, 1H); IR (KBr) ν 3195, 2934, 2836, 1899, 1584, 1488, 1466, 1438, 1427, 1381, 1299, 1237, 1176, 1121, 1020, 1000, 852, 751 cm^{-1}; MS (EI) *m/z* (rel intensity) 340 (M^+, 100).

3.3.2. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(3″-methoxyphenyl)pyrazole (13). Yellow semisolid (148 mg, 83%): ^1H NMR ($CDCl_3$) δ 3.62 (s, 3H), 3.66 (s, 3H), 3.82 (s, 3H), 6.63 (s, 1H), 6.74–6.78 (m, 1H), 6.85 (s, 2H), 7.12–7.26 (m, 3H); IR (KBr) ν 3107, 3000, 2938, 2835, 1590, 1494, 1466, 1430, 1391, 1314, 1233, 1170, 1123, 1039, 1001, 858, 729 cm^{-1}; MS (ES^+) *m/z* (rel intensity) 340 (M^+, 100); HRMS [M^+] for $C_{19}H_{20}N_2O_4$: obsd 340.1425, calcd 340.1423.

3.3.3. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(4″-methoxyphenyl)pyrazole (14). Light yellow solid (80 mg, 48%), mp 230 °C: ^1H NMR ($CDCl_3$) δ 3.84 (s, 3H), 3.85 (s, 3H), 3.86 (s, 6H), 6.84 (s, 1H), 6.98 (d, 1H, J = 8.5 Hz), 7.02 (s, 2H), 7.14 (d, 1H, J = 7.5 Hz), 7.69 (d, 1H, J = 8.5 Hz), 7.72 (d, 1H, J = 8.5 Hz); IR (KBr) ν 3392, 2948, 1171, 1459, 1376, 1215, 1167, 761 cm^{-1}; MS (EI) *m/z* (rel intensity) 340 (M^+, 100).

3.3.4. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(2″,5″-dimethoxyphenyl)pyrazole (15). Yellow solid (81 mg, 53%), mp 99 °C: ^1H NMR ($CDCl_3$) δ 3.84 (s, 3H), 3.88 (s, 3H), 3.89 (s, 3H), 3.95 (s, 6H), 6.82 (d, 1H, J = 8.5 Hz), 6.87 (s, 1H), 6.96 (d, 1H, J = 8.5 Hz), 7.11 (s, 2H), 7.69 (s, 1H); IR (KBr) ν 3341, 2999, 2936, 2833, 1622, 1591, 1508, 1461, 1264, 1233, 1124, 1020, 808, 756 cm^{-1}; MS (EI) *m/z* (rel intensity) 370 (M^+, 100).

3.3.5. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(3″,4″-dimethoxyphenyl)pyrazole (16). Yellow solid (96 mg, 78%), mp 68 °C: ^1H NMR ($CDCl_3$) δ 3.65 (s, 6H), 3.66 (s, 3H), 3.82 (s, 3H), 3.84 (s, 3H), 6.56 (s, 1H), 6.68–6.71 (m, 1H), 6.87 (s, 2H), 7.14–7.18 (m, 2H); IR (KBr) ν 3331, 3123, 3009, 2936, 2833, 1586, 1508, 1467, 1430, 1249, 1129, 1025, 865, 756 cm^{-1}; MS (EI) *m/z* (rel intensity)

370 (M^+, 100); HRMS [M^+] for $C_{20}H_{22}N_2O_5$: obsd 370.1523, calcd 370.1529.

3.3.6. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(3″,4″,5″-trimethoxyphenyl)pyrazole (17). Brown-yellow solid (82 mg, 43%), mp 200 °C: ^{1}H NMR ($CDCl_3$) δ 3.73 (s, 12H), 3.85 (s, 6H), 6.59 (s,1H), 6.90 (s, 4H); IR (KBr) ν 3130, 3005, 2938, 2834, 1591, 1503, 1472, 1429, 1389, 1328, 1241, 1187, 1166, 1128, 1005, 864, 838, 754 cm^{-1}; MS (EI) *m/z* (rel intensity) 400 (M^+, 100); HRMS [M^+] for $C_{21}H_{24}N_2O_6$: obsd 400.1636, calcd 400.1634.

3.3.7. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(4″-nitrophenyl)pyrazole (18). Yellow solid (174 mg, 64%), mp 192 °C: ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 6H), 3.88 (s, 3H), 6.85 (s, 2H), 6.87 (s, 1H), 7.91 (d, 2H, J = 8.7 Hz), 8.23 (d, 2H, J = 8.7 Hz); IR (KBr) ν 3310, 2925, 2843, 1592, 1517, 1464, 1415, 1341, 1240, 1126, 1000, 855, 754 cm^{-1}; MS (EI) *m/z* (rel intensity) 355 (M^+, 100); HRMS [M^+] for $C_{18}H_{17}N_3O_5$: obsd 355.1170, calcd 355.1168.

3.3.8. 3-(3′,4′,5′-Trimethoxyphenyl)-5-(4″-methoxy-3″-nitrophenyl)pyrazole (20). Yellow solid (0.10 g, 60%), mp 82 °C: ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 3H), 3.84 (s, 6H), 3.92 (s, 3H), 6.85 (s, 1H), 7.00 (s, 2H), 7.16 (d, 1H, J = 9 Hz), 7.98 (dd, 1H, J = 9 Hz, 2.1 Hz), 8.22 (d, 1H, J = 2.1 Hz); IR (KBr) ν 3009, 2936, 2833, 1621, 1581, 1531, 1462, 1353, 1276, 1126, 1009, 755 cm^{-1}; MS (EI) *m/z* (rel intensity) 385 (M^+, 100); HRMS [M^+] for $C_{19}H_{19}N_3O_6$: obsd 385.1273, calcd 385.1274.

3.3.9. 3-(2′,5′-Dimethoxyphenyl)-5-(3″,4″,5″-trimethoxyphenyl)pyrazole (22). Yellow semisolid (225 mg, 94%): ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H), 3.89 (s, 3H), 3.96 (s, 6H), 3.97 (s, 3H), 6.86 (s, 1H), 6.87 (dd, 1H, J = 9.0, 3.0 Hz), 6.94 (d, 1H, J = 9.0 Hz), 7.11 (s, 2H), 7.27 (d, 1H, J = 3.0 Hz); IR (KBr) ν 3714, 2247, 3002, 2939, 2834, 2352, 1589, 1500, 1463, 1227, 1122, 1038, 1002 cm^{-1}; MS (EI) *m/z* (rel intensity) 370 (M^+, 100); HRMS [M^+] for $C_{20}H_{22}N_2O_5$: obsd 370.1529, calcd 370.1529.

3.3.10. 3-(2′,5′-Dimethoxyphenyl)-5-(4″-methoxy-3″-nitrophenyl)pyrazole (23). Brown solid (183 mg, 83%), mp 57 °C: ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H), 3.98 (s, 3H), 4.01 (s, 3H), 6.91 (s, 1H), 7.15 (d, 1H, J = 8.7 Hz), 8.08 (dd, 1H, J = 8.7 Hz, 2.3 Hz), 8.33 (d, 1H, J = 2.3 Hz); IR (KBr) ν 2923, 2843, 1622, 1537, 1493, 1456, 1351, 1279, 1226, 1176, 1041, 1019, 805, 745 cm^{-1}; MS (EI) *m/z* (rel intensity) 355 (M^+, 100); HRMS [M^+] for $C_{18}H_{17}N_3O_5$: obsd 355.1170, calcd 355.1168.

3.3.11. 3-(2′,5′-Dimethoxyphenyl)-5-(4″-nitrophenyl)pyrazole (25). Brown solid (161 mg, 83%), mp 184 °C: ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H), 4.00 (s, 3H), 6.91 (dd, 1H, J = 8.8, 3.0 Hz), 7.00 (d, 1H, J = 8.8 Hz), 7.01 (s, 1H), 7.26 (d, 1H, J = 3.0 Hz), 8.04 (d, 2H, J = 8.5 Hz), 8.30 (d, 2H, J = 8.5 Hz); IR (KBr) ν 2999, 2940, 2833, 1601, 1515, 1338, 1226, 1176, 1109, 1041, 984, 953, 854, 797, 750 cm^{-1}; MS (EI) *m/z* (rel intensity) 355 (M^+, 100); HRMS [M^+] for $C_{18}H_{17}N_3O_5$: obsd 355.1170, calcd 355.1168.

3.4. General preparation of aminopyrazoles (19, 21, 24, 26)

A suspension of a nitro-3,5-diarylpyrazole (20 mg) and 5% Pd/C (10 mg) in THF was hydrogenated at atmospheric pressure and room temperature for 4 h. The catalyst was removed by filtration over Celite and the desired products were obtained by concentration of the filtrate. TLC and 500 MHz ^{1}H NMR analyses indicated that the compounds were homogeneous.

3.4.1. 5-(4″-Aminophenyl)-3-(3′,4′,5′-trimethoxyphenyl)pyrazole (19). Tan solid (20 mg, 95%), mp 90 °C: ^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3H), 3.95 (s, 6H), 6.68 (s, 1H), 6.76 (d, 2H, J = 8.5 Hz), 7.01 (s, 2H), 7.46 (d, 2H, J = 8.5 Hz); IR (KBr) ν 2999, 2935, 1619, 1589, 1506, 1465, 1368, 1238, 1179, 1126, 1001, 833, 756 cm^{-1}; MS (EI) *m/z* (rel intensity) 325 (M^+, 100); HRMS [M^+] for $C_{18}H_{19}N_3O_3$: obsd 325.1430, calcd 325.1426.

3.4.2. 5-(3″-Amino-4″-methoxyphenyl)-3-(3′,4′,5′-trimethoxyphenyl)pyrazole (21). Yellow semisolid (22.6 mg, 78%): ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H), 3.91 (s, 3H), 3.95 (s, 6H), 6.69 (s, 1H), 6.73–6.75 (m, 1H), 6.84–6.86 (m, 1H), 7.01 (s, 2H), 7.03–7.07 (m, 1H); IR (KBr) ν 3368, 2949, 2834, 1620, 1589, 1510, 1458, 1227, 1122, 1028, 907 cm^{-1}; MS (EI) *m/z* (rel intensity) 355 (M^+, 100); HRMS [M^+] for $C_{19}H_{21}N_3O_4$: obsd 355.1538, calcd 355.1532.

3.4.3. 5-(3″-Amino-4″-methoxyphenyl)-3-(2′,5′-dimethoxyphenyl)pyrazole (24). Tan solid (12 mg, 94%), mp 73 °C: ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H), 3.90 (s, 3H), 3.96 (s, 3H), 6.83–6.89 (m, 4H), 6.96–6.98 (m, 2H), 7.22–7.24 (m, 1H); IR (KBr) ν 2931, 2843, 1617, 1498, 1461, 1254, 1227, 1171, 1025, 909, 802, 735 cm^{-1}; MS (EI) *m/z* (rel intensity) 325 (M^+, 100); HRMS [M^+] for $C_{18}H_{19}N_3O_3$: obsd 325.1430, calcd 325.1426.

3.4.4. 5-(4″-Aminophenyl)-3-(2′,5′-dimethoxyphenyl)pyrazole (26). Brown-yellow solid (24 mg, 88%), mp 115 °C: ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H), 3.98 (s, 3H), 6.75 (d, 2H, J = 8.5 Hz), 6.84 (s, 1H), 6.90 (dd, 1H, J = 8.5, 3.0 Hz), 6.97 (d, 1H, J = 8.5 Hz), 7.27 (d, 1H, J = 3.0 Hz), 7.66 (d, 2H, J = 8.5 Hz); IR (KBr) ν 2962, 2843, 1619, 1497, 1453, 1262, 1227, 1179, 1088, 1043, 910, 803, 734 cm^{-1}; MS (EI) *m/z* (rel intensity) 295 (M^+, 100).

4. X-ray crystallography

The molecular structure of derivative **17**, $C_{21}H_{24}N_2O_6$, was determined by X-ray diffraction methods. The substance crystallizes in the monoclinic space group $P2_1$ with a = 14.950(3) Å, b = 43.390(9) Å, c = 14.980(3) Å, β = 116.76(3)°, and Z = 16 refined to an agreement R1 factor of 0.0862. The hydrogen atoms were positioned in ideal positions and refined with the riding model.

Two regions of electron density not related to any of the molecules were refined as partial ethyl acetate molecules. Intramolecular bond distances and angles, along with other crystallographic data, are given as supporting information. The structure of compound **17** has been deposited in the Cambridge Crystallographic Data Centre, reference number CCDC-269383. The structures of compounds **3** and **7** were determined using a similar procedure.

5. Cytotoxicity

All biological solutions were purchased from Atlanta Biologicals, Atlanta. DMSO was purchased from Sigma-Aldrich PLc. The B16 and L1210 cell lines were obtained from the American Type Tissue Culture Collection (ATCC). Cells were cultured using Delbeucco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (10%), Hepes buffer (2 mM), L-glutamine (2 mM), penicillin/streptomycin (50,000 U penicillin, 50,000 μg streptomycin). Cells were maintained at 37 °C in a 5% humidified CO_2 atmosphere in a Revco Ultima II incubator.

Dose–response curves were obtained for each compound using a 72 h continuous exposure MTT assay detailed by Carmichael et al.[24] Cultured cells were counted and suspended in DMEM at concentrations of 4 and 8×10^4 cells/cm^3 for B16 and L1210 cell lines, respectively. Ninety-six-well plates were seeded at concentrations of 4000 and 8000 cells/well for the respective cell lines. Each compound was dissolved in DMSO to produce a 1.75×10^{-2} M stock solution. Serial 1 in 10 dilutions were performed (with the addition of DMEM) to produce final ligand concentrations ranging from 1.0×10^{-4} to 1.0×10^{-12} M. The plates were treated with the compound dilutions (5 μL/well) in quadruplicate.

Plates were then incubated at 37 °C in a 5% humidified CO_2 atmosphere for 72 h. MTT in PBS (5 mg/cm^3) was added (20 μL/well) and the plates incubated as before for a further 4 h. Acid IPA solution (16 μL, 12.1 M HCl in 5 cm^3 IPA) was then added (100 μL/well). Following mixing, the plates were analyzed using a Bio-Rad 680 microplate reader at 570 nm. Dose–response curves were plotted using the average of the quadruplicate results. IC_{50} values were calculated at 50% of the cell growth using at least two separate replicates.

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