Pergamon

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Bioorganic & Medicinal Chemistry 11 (2003) 913–929

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis, Characterization and In Vitro Anti-invasive Activity Screening of Polyphenolic and Heterocyclic Compounds

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Received 25 September 2002; accepted 21 October 2002

Dedicated to the cherished memory of our collaborator and friend (Late) Professor Sukant K. Tripathy

Abstract—Invasion is the hallmark of malignant tumors, and is responsible for the bad prognosis of the untreated cancer patients. The search for anti-invasive treatments led us to screen compounds of different classes for their effect in an assay for invasion. Thirty-nine new compounds synthesized in the present study along with 56 already reported compounds belonging mainly to the classes of lactones, pyrazoles, isoxazoles, coumarins, desoxybenzoins, aromatic ketones, chalcones, chromans, isoflavanones have been tested against organotypic confronting cultures of invasive human MCF-7/6 mammary carcinoma cells with embryonic chick heart fragments in vitro. Three of them (a pyrazole derivative, an isoxazolylcoumarin and a prenylated desoxybenzoin) inhibited invasion at concentrations as low as 1 μM; instead of occupying and replacing the heart tissue within 8 days, the MCF-7/6 cells grew around the heart fragments and left it intact, when treated with these compounds. At the anti-invasive concentration of 1 μM, the three compounds did not affect the growth of the MCF-7/6 cells, as shown in the sulforhodamine B assay. Aggregate formation on agar was not stimulated by any of the three anti-invasive compounds, making an effect on the E-cadherin/catenin complex improbable. This is an invasion suppressor that can be activated in MCF-7/6 cells by a number of other molecules. Our data indicate that some polyphenolic and heterocyclic compounds are anti-invasive without being cytotoxic for the cancer cells.
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Introduction

Growth and invasion are tumor activities that are responsible for the fatal outcome of untreated cancer patients. Oncologists nowadays possess surgery, radiotherapy, chemotherapy and immunotherapy as efficient tools to tackle tumor growth. Invasion, however, is a more resistant problem than growth, and anti-invasive agents are sadly lacking in clinical practice.[1] Progress in this field can be expected from a better knowledge of the molecular mechanisms of invasion, from the development of relevant invasion models in vitro and from the

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PII: S0968-0896(02)00539-4

synthesis or isolation of new compounds as candidates for anti-invasive drugs. Among alkaloids and polyphenolics, a number of compounds have already been detected that could interfere with cellular protein complexes that are implicated in invasion[2–4] although the poor specificity of their targets for tumor cells often limited their application in medicine.

Because there is considerable need in oncology for anti-invasive agents and very few agents of that kind are available, no chemical rationale for directed screening of compounds towards this goal is prevailing. As a part of our continued search for new anti-invasive agents, we tested ninety-five compounds belonging to lactones, pyrazoles, isoxazoles, coumarins, desoxybenzoins, aromatic ketones, chalcones, chromans, isoflavanones and other classes. These compounds were tested in an organotypic assay for invasion. Here, a living host tissue fragment was confronted with invasive cancer cells, mimicking the elements of the micro-ecosystem encountered in human tumors.[5] Human MCF-7/6 mammary carcinoma cells[6] were used in this assay, because they can invade the normal tissue fragment within 1 week, and also because their invasiveness was proven to be sensitive to polyphenolics and hormones.

Results

Preparation and characterization of compounds

The structural formulae of the compounds studied in this report are shown in Figure 1. The lactones **1** and **2** were synthesised by our earlier published procedure by condensing the corresponding acetophenone with ethyl 2-cyano-3,3-bis(thiomethyl)propenoate in the presence of KOH in DMF.[7] These lactones have been used as starting materials for the synthesis of the pyrazolyl acetonitriles **3**[7] and **4**[7] by treating the corresponding lactone with hydrazine hydrate in methanol under reflux conditions. However, the *N*-phenyl/aryl substituted pyrazolyl acetonitriles **5–16** were obtained by condensing phenyl/aryl hydrazine hydrate with lactones of the type **1** and **2**; the compounds **5** and **8–16** have been prepared for the first time and characterized completely from their spectral data, however the compounds **6**[8,9] and **7**[8] have been reported earlier. During the synthesis of these pyrazolyl acetonitriles, we isolated in many cases, the 3-amino-2,6-diaryl-4-oxo-4*H*-pyrano-[4,3-*c*]pyrazoles **17–26** as co-products. Of these, **17** and **18** have been reported earlier,[8] while **19–26** are not known in literature and are being reported for the first time.

The compounds **27–37**[10] were obtained by condensation of pyrazolyl acetonitriles with pyrrole 2-carboxaldehyde in the presence of sodium-*t*-butoxide in ethanol, both the *E* and *Z* isomers could be obtained by varying the reaction conditions.[10] However, condensation of 3,4-difluorobenzaldehyde with respective phenylpyrazolyl acetonitriles under identical conditions afforded the *trans* (*E*) condensation products **38–42**, these compounds have been prepared for the first time and were well characterized from their spectral data. The compounds **43–45**, **49** and **50** were synthesised by the earlier published procedure,[11] the same procedure was followed for the synthesis of **46–48** and **51**. Of these, **46**, **48** and **51** are new in literature, however, **47**[7] has been reported earlier.

The isoxazolylcoumarins **52–54** were synthesised by treating the corresponding aryl-cyanomethylisoxazoles with salicylaldehyde in ethanol in the presence of sodium hydroxide, all the three are new compounds. The coumarins **55**,[12] **56**[13] and **57**[14] were synthesised by the well known Pechmann condensation reaction.[15] The novel new coumarins **58–60** were prepared by butanoylation of 7,8-dihydroxy-4-methylcoumarin,[14] 7-hydroxy-4-methylcoumarin[16] and 5,7-dihydroxy-4-methylcoumarin,[17] respectively using butyric anhydride and pyridine, while **61** was prepared by the methylation of 5,7-dimethoxy-6-hydroxy-4-methylcoumarin (**56**)[13] using dimethyl sulphate and potassium carbonate in anhydrous acetone and is reported for the first time. The amides **62**,[18] **63**[18] and **64–69** were prepared by our earlier published procedure.[19]

The compounds **70–72** and **78**[20] were prepared by prenylation of 2,4-dihydroxyphenyl benzyl ketone and 4-hydroxyacetophenone, respectively using 2-methylbut-3-en-2-ol and BF_3-etherate.[21] The compounds **76** and **77** were obtained according to the method of Lars et al.[22]

Table 1. Effect of polyphenolic and heterocyclic compounds on invasion of MCF-7/6 cells in vitro

	μM			μM			μM	
Compound No.	10	1	Compound No.	10	1	Compound No.	10	1
1	−	0	**33**	+	−	**65**	−	0
2	−	0	**34**	+	−	**66**	−	0
3	+	−	**35**	+	−	**67**	−	0
4	−	0	**36**	+	−	**68**	−	0
5	−	0	**37**	+	−	**69**	−	0
6	+	−	**38**	−	0	**70**	+	−
7	+	−	**39**	+	+	**71**	+	+
8	−	0	**40**	−	0	**72**	+	−
9	−	0	**41**	+	−	**73**	−	0
10	−	0	**42**	+	−	**74**	+	−
11	+	−	**43**	+	−	**75**	−	0
12	+	−	**44**	−	0	**76**	+	−
13	+	−	**45**	−	0	**77**	−	0
14	−	0	**46**	+	−	**78**	−	0
15	−	0	**47**	−	0	**79**	−	0
16	0	0	**48**	−	0	**80**	0	0
17	−	0	**49**	−	0	**81**	0	0
18	−	0	**50**	−	0	**82**	0	0
19	−	0	**51**	−	0	**83**	0	0
20	−	0	**52**	−	0	**84**	−	0
21	−	0	**53**	+	+	**85**	−	0
22	−	0	**54**	−	0	**86**	+	−
23	−	0	**55**	−	0	**87**	−	0
24	−	0	**56**	−	0	**88**	+	−
25	+	−	**57**	−	0	**89**	−	0
26	0	0	**58**	−	0	**90**	−	0
27	+	−	**59**	+	−	**91**	−	0
28	+	−	**60**	−	0	**92**	+	−
29	+	−	**61**	−	0	**93**	−	0
30	+	−	**62**	−	0	**94**	+	−
31	−	−	**63**	−	0	**95**[29]	+	−
32	+	−	**64**	−	0			

+, anti-invasive; −, not anti-invasive; 0, not tested.

The benzopyran **73** was obtained by cyclization of prenyl group in **70** with adjacent hydroxyl group using formic acid.[23] The compound **79** was prepared by the reaction of 4-hydroxyacetophenone with isoprene by the method of Clemo and Ghatge.[24] However, on subjecting the compound **70** to oxidative cyclisation using *m*-chloroperoxybenzoic acid (*m*-CPBA),[25] **74** was obtained, which on subsequent methylation using dimethyl sulphate and potassium carbonate in anhydrous acetone yielded **75**. The compounds **70–75** are reported for the first time. However, the ketones **80–85**,[25,26] the quinone **86**[27] and the chalcones **87–92**[28] were synthesised by following the indicated literature methods, while the compounds **93–94** were obtained from the collection of (Late) Professor T.R. Seshadri, FRS in Delhi (India). The structures of all the thirty nine new compounds synthesized in this study have been established unambiguously on the basis of their spectral data, that is ^{1}H and ^{13}C NMR, IR, UV, MS and HR-MS.

Invasion

Table 1 summarizes the results of the assays for invasion. In control cultures treated with the solvent only, the MCF-7/6 cells had invaded the PHF after 8 days of incubation. In histological sections of these cultures, only the remnants of the heart tissue could be discerned, as visualized by the haematoxylin-eosin staining. Massive replacement of the heart fragment was evident from selective immunohistochemistry of the MCF-7/6 cells (Fig. 2A and B). Many compounds showed no inhibition of invasion, and histologically their cultures were

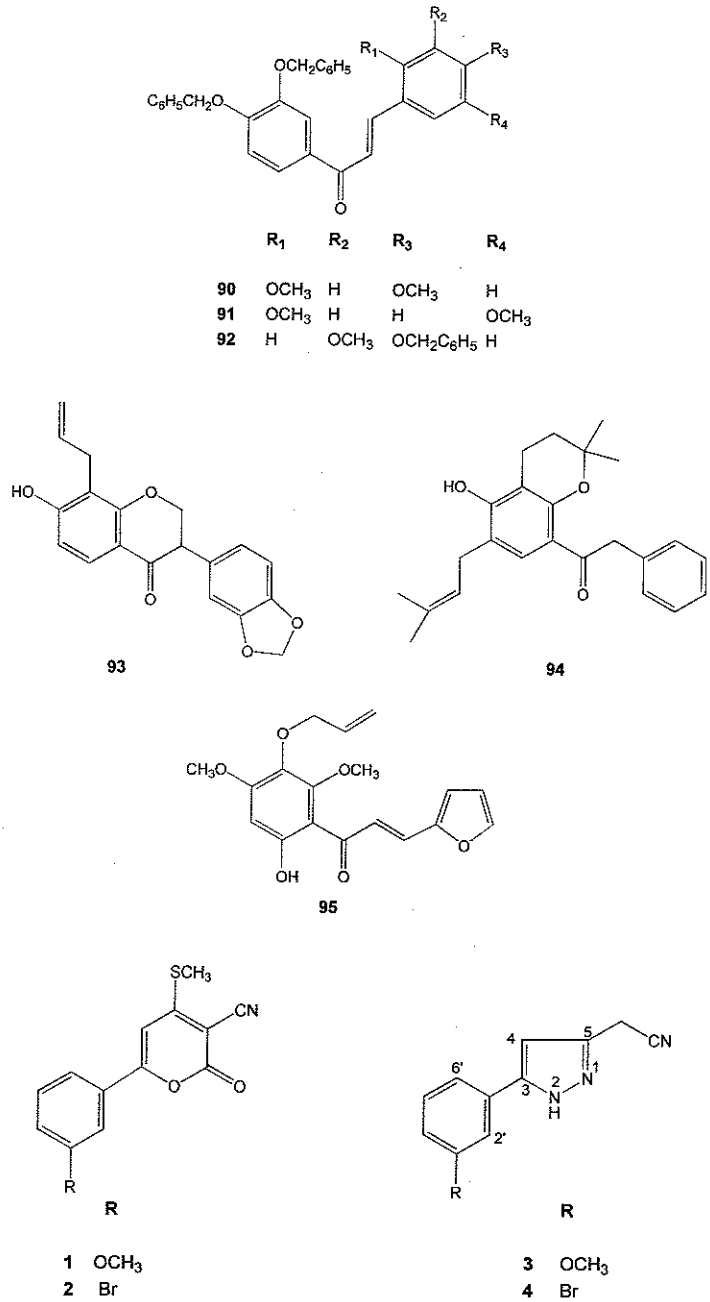
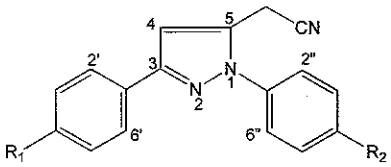
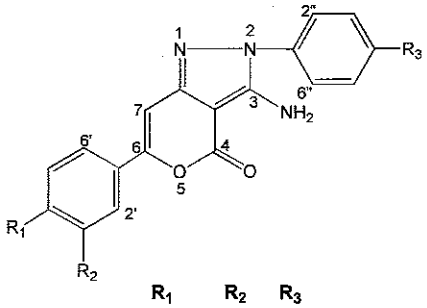


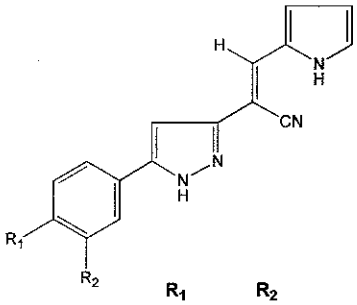
Figure 1. Structures of compounds tested as anti-invasive agents in vitro.



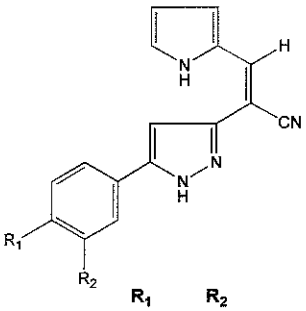
	R_1	R_2
5	CH_3	F
6	CH_3	H
7	Cl	H
8	F	H
9	OCH_3	H
10	H	F
11	Br	Br
12	Br	F
13	OCH_3	F
14	Cl	F
15	F	F
16	OCH_3	Cl



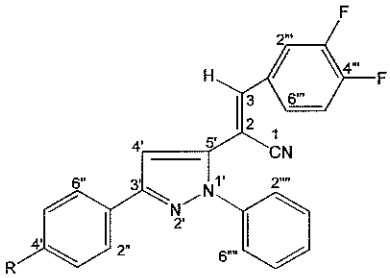
	R_1	R_2	R_3
17	Br	H	H
18	OCH_3	H	H
19	F	H	H
20	-O-CH_2-O-		H
21	CH_3	H	F
22	Cl	H	F
23	F	H	F
24	F	H	Cl
25	OCH_3	H	F
26	OCH_3	H	Cl



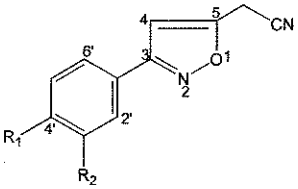
	R_1	R_2
27	Cl	H
28	OCH_3	H
29	F	H
30	H	H
31	CH_3	H
32	Br	H
33	H	Br



	R_1	R_2
34	Cl	H
35	OCH_3	H
36	F	H
37	H	OCH_3

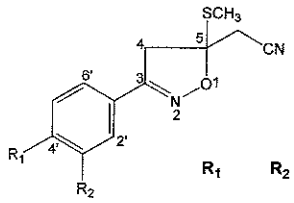


	R
38	H
39	Cl
40	Br
41	CH_3
42	OCH_3

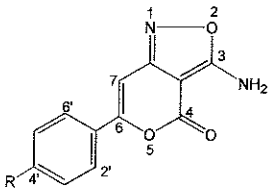


	R_1	R_2
43	Cl	H
44	CH_3	H
45	Br	H
46	-O-CH_2-O-	
47	OCH_3	H

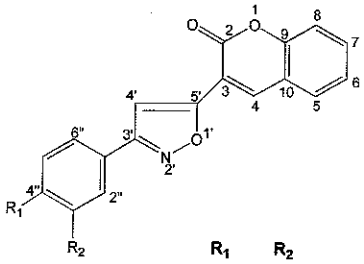
Figure 1. (Continued).



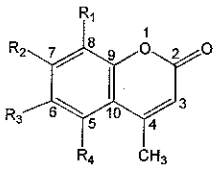
	R_1	R_2
48	-O-CH_2-	O-
49	Cl	H



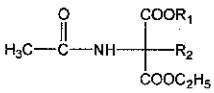
	R
50	Cl
51	F

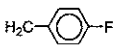
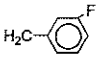
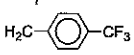
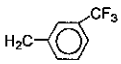
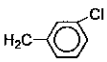


	R_1	R_2
52	Cl	H
53	CH_3	H
54	-O-CH_2-O-	



	R_1	R_2	R_3	R_4
55	$OCOCH_3$	$OCOCH_3$	H	H
56	H	OCH_3	OH	OCH_3
57	H	$OCOCH_3$	H	H
58	$OCO(CH_2)_2CH_3$	$OCO(CH_2)_2CH_3$	H	H
59	H	$OCO(CH_2)_2CH_3$	H	H
60	H	$OCO(CH_2)_2CH_3$	H	$OCO(CH_2)_2CH_3$
61	H	OCH_3	OCH_3	OCH_3



	R_1	R_2
62	H	H
63	C_2H_5	H
64	C_2H_5	
65	C_2H_5	
66	C_2H_5	
67	C_2H_5	
68	C_2H_5	
69	C_2H_5	

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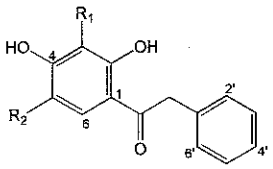
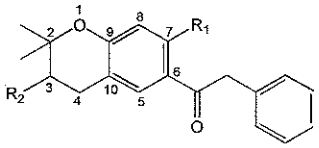
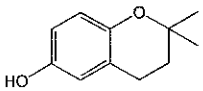


	R_1	R_2
70	H	Pr
71	Pr	H
72	Pr	Pr

Pr = $-CH_2-CH=C(CH_3)_2$



	R_1	R_2
73	OH	H
74	OH	OH
75	OCH_3	OH



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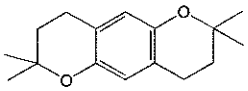

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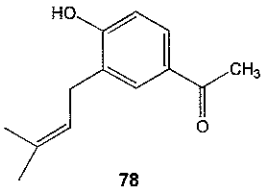

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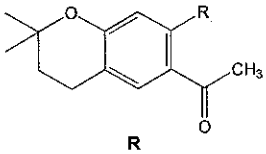
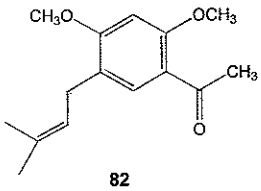


R

	R
79	H
80	OH
81	OCH_3



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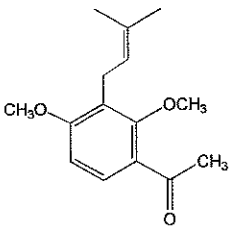

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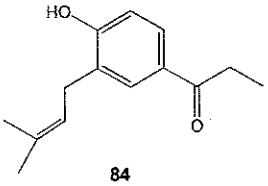

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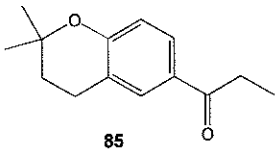

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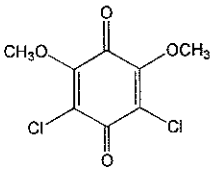

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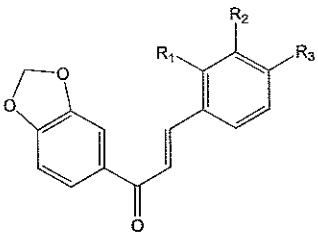

	R_1	R_2	R_3
87	OCH_3	H	OCH_3
88	OCH_3	OCH_3	H
89	H	OCH_3	H

Figure 1. (Continued).

similar to controls. Other compounds were anti-invasive at the concentration of 10 μM. Three out of the 95 compounds (**39**, **53** and **71**) however, inhibited invasion of MCF-7/6 cells at a concentration as low as 1 μM. At 0.1 μM, none of the compounds was active. As shown in Figure 2D–F, cultures treated with anti-invasive compounds showed an intact PHF surrounded by MCF-7/6 cells.

Growth

No growth inhibition of the MCF-7/6 cells could be observed on histological sections from confronting cultures treated with the anti-invasive compounds **39**, **53** or **71**. This was in contrast with the effect of the chalcone **95** (a typical anti-invasive compound studied by us earlier[29]), which has now been seen to have in addition a

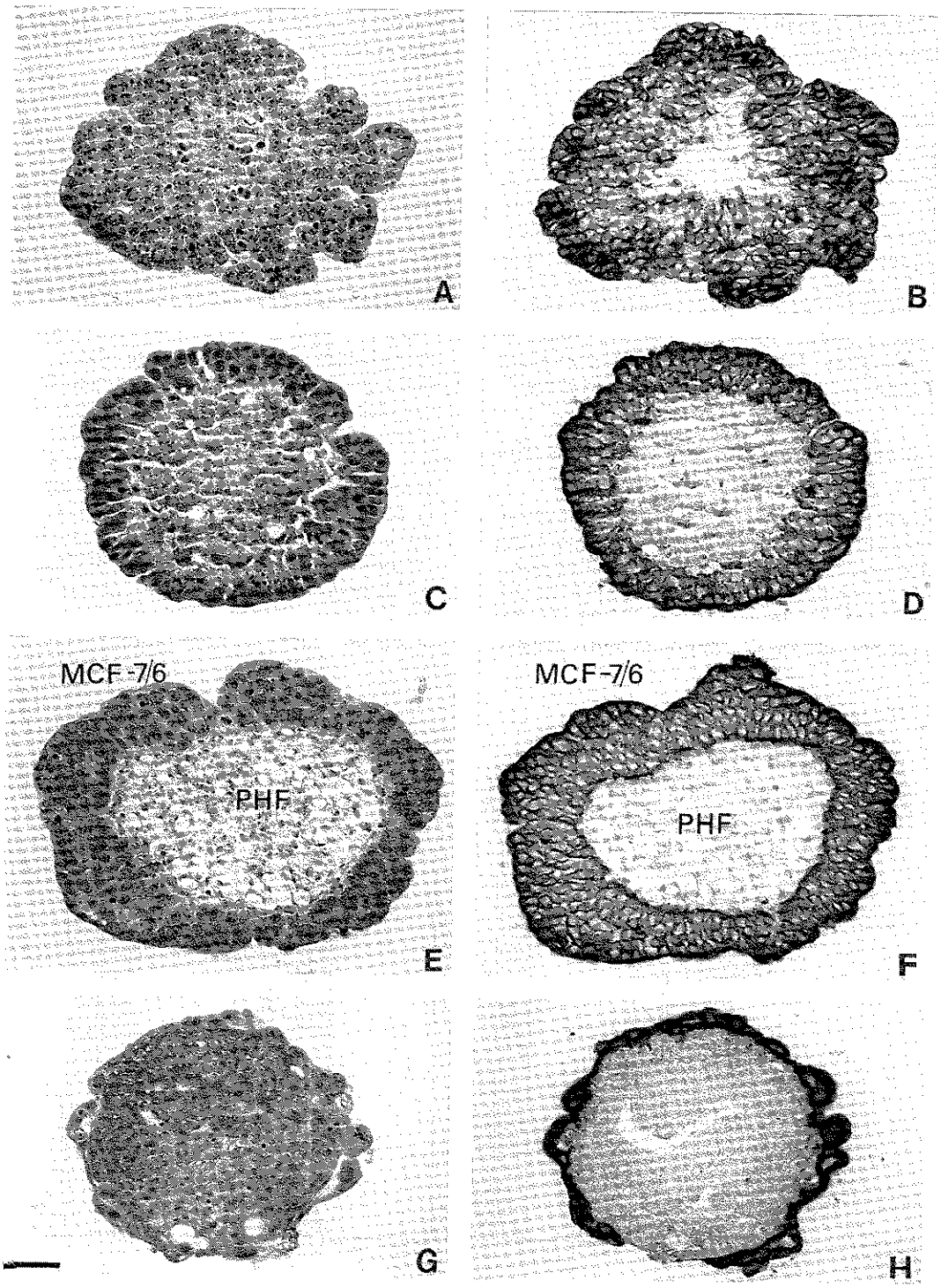

Figure 2. Light micrographs of sections from 8-day-old confronting cultures between precultured heart fragments (PHF) and MCF-7/6 cells. Solvent-treated (0.1% DMSO) confrontations (A and B), that show invasion of MCF-7/6 cells, are compared with confrontations treated with 10 μM of the compounds **39** (C and D), **53** (E and F) and **95** (G and H), that show absence of invasion. The sections on the left panels were stained with haematoxylin-eosin; in the right panels MCF-7/6 antigens were revealed immunohistochemically and appear dark. Scale bare = 50 μm.

remarkable growth inhibition activity on MCF-7/6 cells (Fig. 2G and H). In the sulforhodamine B (SRB) assay, no growth inhibition (not more than 20%) by the anti-invasive compounds **39**, **53** and **71** on MCF-7/6 cells was noticed, neither at 10 μM nor at 1 μM concentration (Fig. 3). The compound **95**, however, which was used as a positive control for growth inhibition (pos) on these cells, reduced growth (by more than 70%) in this assay at 10 μM concentration (Fig. 3). This control was necessary because none of the present anti-invasive compounds affected growth of MCF-7/6 cells at the tested three concentrations.

Cell aggregation

The poor capacity of MCF-7/6 cells to form cell aggregates was evident from untreated and solvent treated cultures; only small and irregular aggregates were formed on semi-solid agar. Treatment with the anti-invasive compounds **39**, **53** or **71** at 10 or 1 μM concentrations did not alter the aggregation pattern of the cells (data not shown). As a positive control for induction of cell aggregation, insulin-like growth factor-I (IGF-I) at 500 ng/mL was applied; this treatment induced the formation of large spheroidal aggregates.[30]

Relationship of anti-invasive activity of compounds with their structures

All the compounds were first tested for anti-invasive activity at concentration of 100 μM, those showing positive results were further tested at lower concentrations. Thirty-four compounds, that is **3**, **6**, **7**, **11–13**, **25**, **27–30**, **32–37**, **39**, **41–43**, **46**, **53**, **59**, **70–72**, **74**, **76**, **86**, **88**, **92**, **94** and **95** out of 95 exhibited anti-invasive activity at 10 μM concentration on MCF-7/6 cells in the chick heart invasion assay (Table 1). A majority of compounds found active at 10 μM concentration are either pyrazole derivatives or aromatic ketones, mainly desoxybenzoins and chalcones. Thirteen out of 16 pyrazolylacrylonitriles, namely compounds **27–30**, **32–37**, **39**, **41** and **42** out of **27–42** exhibited anti-invasive activity at 10 μM concentration on MCF-7/6 cells in the chick heart invasion assay; one of them, that is (*Z*)-2-[3-(4-chlorophenyl)-1-phenylpyrazol-5-yl)-3-(3,4-difluorophenyl)acrylonitrile (**39**) was also active at 1 μM concentration. Out of the five pyrazolyl acrylonitriles **38–42** with 3,4-difluorophenyl substituent at C-3 position, three compounds **39**, **41** and **42** exhibited antiinvasive activity at 10 μM concentration and among the three compounds **39** was found active at 1 μM concentration. This revealed that difluorophenyl moiety at the C-3 position together with the 4-chlorophenyl moiety at the C-3 -position of pyrazole in compound **39** enhances the anti-invasive activity of the acrylonitrile. Three compounds, that is **43**, **46** and **53** out of twelve isoxazole derivatives **43–54** evaluated in this assay, exhibited anti-invasive activity at 10 μM concentration; an isoxazolylcoumarin out of these three, that is 3-[3-(4-methylphenyl)isoxazol-5-yl]-2*H*-1-benzopyran-2-one (**53**) was also active at 1 μM concentration. Highest activity of isoxazolylcoumarin **53** indicates that the construction of a coumarin moiety involving the C-5 cyanomethyl group of the isoxazole enhances the anti-invasive activity of the compound by manifold. Five prenylated desoxybenzoines, that is **70–72**, **74** and **94** out of seven compounds of this class, namely **70–75** and **94** evaluated in this assay, exhibited activity at 10 μM concentration; 2,4-dihydroxy-3-prenyldesoxybenzoin (**71**) was also active at 1 μM concentration. This indicates that

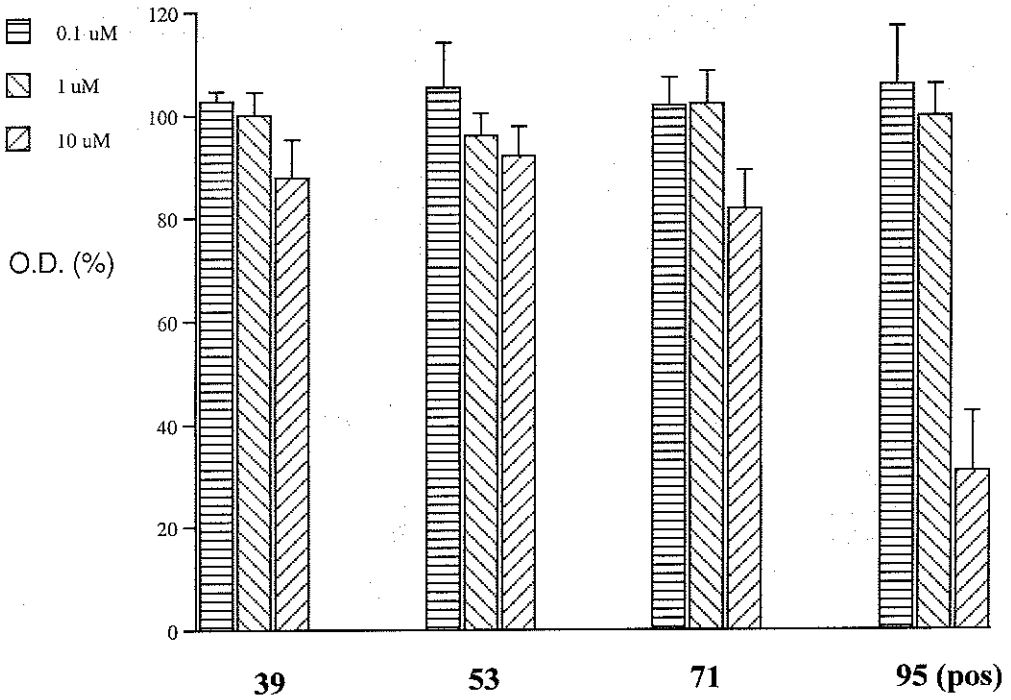

Figure 3. Effect of three anti-invasive compounds in the sulforhodamine B assay with MCF-7/6 cells grown in microtiter plates for 3 days. The cells were treated with 0.1, 1 or 10 μM of each compound. Treatment with compound **95** (pos) is included as a positive control, and results are presented as a percentage of solvent-treated controls (mean + standard deviation).

prenylated desoxybenzoins are potential anti-invasive compounds. The close inspection of the structures of these desoxybenzoins also revealed that the compounds with prenyl group present in the open form are more active, particularly when the prenyl group is flanked by two hydroxyl groups as in **71**, which had the maximum anti-invasive activity of the compounds of this class. Together with these pyrazole and isoxazole derivatives, and desoxybenzoins, one coumarin derivative **59** out of seven (**55–61**) and three chalcones **88**, **92** and **95** out of seven (**87–92** and **95**) evaluated in this assay, showed anti-invasive activity at 10 μM concentration. The three most active compounds, that is **39**, **53** and **71** that are active at 1 μM concentration, do not show any anti-invasive activity at 0.1 μM concentration.

Discussion

From the 95 compounds, **39**, **53** and **71** were found to possess an anti-invasive activity on MCF-7/6 cells in the chick heart invasion assay at a concentration as low as 1 μM. These three novel compounds have been fully characterized. The molecular formula $C_{24}H_{14}N_3F_2Cl$ of (*Z*)-2-[3-(4-chlorophenyl)-1-phenylpyrazol-5-yl]-3-(3,4-difluorophenyl) acrylonitrile (**39**) was determined on the basis of its EI–MS, HR-MS and hydrogen and carbon counts form its ^{1}H and ^{13}C NMR spectra. The absorption at 2215 cm^{-1} in the IR spectrum of **39** revealed the presence of –C≡N group in the compound. Two characteristic singlets, each for one proton appeared at δ 6.93 and 7.03 for the C-3H and C-4′H, respectively and the 12 aromatic protons present in compound **39** resonated between δ 7.20 to 7.82 as four multiplets and a doublet in its ^{1}H NMR spectrum. The ^{13}C NMR spectrum of compound **39** showing signals for all the 24 different carbon atoms was in full agreement with its structure. The molecular formula $C_{19}H_{13}O_3N$ of the isoxazolylcoumarin **53** was determined on the basis of its EI–MS and hydrogen and carbon counts from its ^{1}H and ^{13}C NMR spectra. The absorption band at 1740 cm^{-1} in the IR spectrum of **53** revealed the presence of a lactone carbonyl group of the coumarin moiety. The singlet at δ 2.40 integrating for three protons revealed the presence of the methyl group at the C-4″ position. The characteristic C-4H of the coumarin moiety and the C-4′H of the isoxazole moiety appeared at δ 8.01 (s) and 7.59 (s), respectively in the ^{1}H NMR spectrum of isoxazolylcoumarin **53**, which also exhibited resonances due to eight aromatic protons as two multiplets for two protons each between δ 7.15–7.21 and 7.39–7.49, and two doublets for two protons each at δ 7.28 and 7.78 (J = 8 Hz each). The peaks in the ^{13}C NMR spectrum of the isoxazolylcoumarin **53** were in full agreement with its structure. The molecular formula $C_{19}H_{20}O_3$ of 2,4-dihydroxy-3-prenyldesoxybenzoin (**71**) was determined on the basis of its EI–MS, HR-MS and hydrogen and carbon count from its ^{1}H and ^{13}C NMR spectra. The IR and UV spectra of **71** revealed the presence of carbonyl and hydroxyl groups in the compound. The ^{1}H NMR spectrum of **71** exhibited the characteristic resonances for the nine protons of the prenyl group at δ1.81 (6H), 3.42 (2H) and 5.24–5.27 (1H). The two *ortho*-coupled protons C-5H and C-6H resonated at δ 6.36 (d, J = 8.85 Hz) and 7.64 (d, J = 8.88 Hz), respectively. The characteristic peaks for the chelated hydroxyl group and the benzylic protons appeared as two singlets at δ 13.00 and δ 4.20, respectively, while the C-4 OH group appeared at δ 6.28 as a singlet, the five aromatic protons at C-2′–C-6′ appeared as a multiplet between δ 7.22 and 7.35. The ^{13}C NMR spectrum of **71** was in full agreement with the structure.

In additional experiments, we could demonstrate that these compounds did not inhibit the growth of the tumor cells, and that they did not exert their effect through an activation of the MCF-7/6 E-cadherin/catenin complex. In the same invasion assay, a number of anti-invasive agents have previously been picked up that inhibited both invasion and growth of the tumor cells. One type of such compounds indeed interfered with a molecular target that is common in both mitosis and directional migration, such as the vinca alkaloids, that bind to tubulin, and thus prevent the assembly of both the mitotic spindle and of the cytoplasmic microtubule complex.[2] Another type of compounds was cytotoxic, and the anti-invasive effect was the result of a reduced number of intrinsically invasive cells in the confronting cultures. These compounds were of interest to the oncologist, provided the cytotoxicity was selective for the tumor cells and not for the normal tissues.[4,31] The anti-invasive compounds in the present study (**39**, **53** and **71**), however inhibited only invasion without affecting growth, which excludes that they due their activity to cytotoxicity. Such selective inhibitors of invasion are interesting tools to dissect targets implicated in the mechanisms of invasion from those implicated in growth.

The E-cadherin/catenin complex is located at the cell membrane of normal epithelial cells, and this cell–cell adhesion complex prevents epithelia to grow beyond their normal tissue boundaries.[32] In invasive tumors derived from epithelial tissues, the expression or the function of the E-cadherin/catenin complex are downregulated.[33] The complex in MCF-7/6 cells is inactive, but sensitive to functional upregulation by a number of compounds that showed to possess an anti-invasive activity.[34] Tangeretin, a citrus methoxyflavone, for instance was able to increase E-cadherin-dependent cell–cell adhesion between MCF-7/6 cells and to inhibit their invasion in the chick heart invasion assay.[3] This mechanism of action could be excluded for the three anti-invasive compounds in this study (i.e., compounds **39**, **53** and **71**), since they did not stimulate cell aggregation in vitro.

A number of other flavonoids and polyphenolics were shown to inhibit invasion in vitro[35] and artificial metastasis in vivo.[36] Some anti-invasive flavonoids, such as 3,7-dimethoxyflavone[37] and (+)-catechin[38] appear to act via targets in the normal tissues confronted by the tumor cells. (+)-Catechin, for instance, binds to laminin, an extracellular matrix protein.[39] Among the prenylatedchalcones, anti-invasive congeners were found that were selectively cytotoxic for the MCF-7/6 cells,

and did not affect the viability of the chick heart host fragment.[4]

Our results with the anti-invasive compounds of this study (**39**, **53** and **71**) emphasize that, although the mechanisms of action are not elucidated, tumor invasion can be tackled via several targets that may serve as many entries for therapeutic rationales. The present study has revealed that compounds belonging to the class of pyrazolylacrylonitriles, that is **27–42** are more likely to exhibit anti-invasive activity against MCF 7/6 cells. The phenyl substituents at N-1 position in the pyrazole and at C-3 position in the acrylonitrile moiety enhance the anti-invasive activity of compounds of this class as (*Z*)-2-[3-(4-chlorophenyl)-1-phenylpyrazol-5-yl]-3-(3,4-diffluorophenyl)acrylonitrile (**39**) was found active even at 1 μM concentration. Similarly, presence of the coumarin moiety at the C-5 position together with a C-4 substituted phenyl group at the C-3 position on an isoxazole nucleus enhance the anti-invasive activity of compounds of this class, thus 3-[3-(4-methylphenyl) isoxazol-5-yl]-2*H*-1-benzopyran-2-one (**53**) was found active even at 1 μM concentration among the 12 isoxazole derivatives **43–54** evaluated for their anti-invasive activity. The desoxybenzoins with *C*-prenyl group, both in the open chain and cyclised form have been identified as a potent class of anti-invasive compounds. The presence of a prenyl group at the C-3 position on a desoxybenzoin nucleus enhances the anti-invasive activity, thus 2,4-dihydroxy-3-prenyldesoxybenzoin (**71**) exhibits activity at 1 μM concentration against MCF-7/6 cells. Based upon these studies, further work is in progress to identify a non-toxic, potent compound active at very low concentrations to provide leads towards new therapeutic agents in cancer research.

Conclusion

Out of the 95 compounds belonging to the different classes, which were evaluated for their anti-invasive activity against invasive human MCF-7/6 mammary carcinoma cells in confrontation with embryonic chick heart fragment in vitro, three lead compounds, namely **39**, **53** and **71** based upon the pyrazole, isoxazole and desoxybenzoin nucleus, respectively were identified. These inhibited the invasion of MCF-7/6 cells on embryonic chick heart fragment at concentrations as low as 1 μM. Further studies revealed that these compounds are anti-invasive without being cytotoxic to cancer cells. The results of the present study may find a lead towards the development of new therapeutic agents to fight cancer.

Experimental

Cells

MCF-7/6 cells, a variant of the MCF-7 cell family were obtained from Dr. Henri Rochefort, Unite d'Endocrinologie Cellulaire et Moleculaire, Montpellier, France. MCF-7 cells were originally established from a pleural effusion of a breast adenocarcinoma patient.[6] These cells, whose identity was confirmed in our laboratory,[40] are invasive both in vitro[41] and in vivo.[42] MCF-7/6 cells were maintained in 25 cm^2 Falcon tissue culture flasks (Becton Dickinson, Europe, Meylan, France) in a mixture of Dulbecco's modification of Earle's Medium and Ham F12 (50:50; Flow, Irvine, UK), supplemented with 0.05% glutamine (w/v), 250 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericine B and 10% fetal bovine serum.

Assay for invasion

The assay consists of three-dimensional confrontations between tumour cells and normal tissue.[43] Fragments of 9-day-old embryonic chick heart were precultured and selected having a diameter of 0.4 mm. These precultured heart fragments (PHF) were confronted individually with an aggregate (diameter of 0.2 mm) of MCF-7/6 cells, first on top of semi-solid agar overnight to allow attachment, and subsequently in liquid medium for eight days in suspension culture. The cultures were then fixed and embedded individually in paraffin, and serially sectioned for histological analysis. In order to evaluate the interaction between the MCF-7/6 cells and PHF, the sections were stained with haematoxylin-eosin or with an immunohistochemical technique to reveal MCF-7 antigens with the 5D10 monoclonal antibody.[44] Occupation and destruction of the PHF was considered as typical for invasion, while growth around the PHF was scored as absence of invasion.

Treatments

All compounds were dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 10^{-1} M from which further dilutions in culture medium were prepared. For each compound, at least two confrontations were treated in suspension with 10 μM for 8 days. If this concentration appeared to be anti-invasive or cytotoxic, lower concentrations (1 or 0.1 μM) were applied. Control cultures were treated with solvent alone (DMSO at corresponding concentrations).

Assay for growth

MCF-7/6 cells were cultured and treated in Nunclon microtitre plates (Nunc, Roskilde, Denmark) at an initial concentration of 1.5×10^6 cells/well for three days. They were fixed with 50% trichloroacetic acid in water at 2 °C for 1 h, and stained with 0.4% sulforhodamine B (SRB) in 1% glacial acetic acid at room temperature for 30 min.[45] Protein-bound stain was solubilized and read at 490 nm with a ν_{max} ELISA reader (Molecular Devices, Palo Alto, CA, USA). The correlation with cell number was initially confirmed by cell counts using a Bruker counting chamber. At least 12 cultures were analysed for each treatment.

Assay for cell aggregation

Confluent monolayers of MCF-7/6 cells were detached, and 2×10^4 cells were seeded in 200 μL medium on

solidified agar (Difco, Detroit, MI, USA) in a microtiter plate (Nunc), and treated with the test agents.[30] Aggregate formation was evaluated under an inverted microscope after 24 h of incubation.

Physical and spectral data of new compounds

[1-(4-Fluorophenyl)-3-(4-methylphenyl)pyrazol-5-yl]acetonitrile (5). White solid, mp 118–119 °C, IR (KBr): 3100, 2920, 2275, 1610, 1520, 1440, 1370, 1235, 1165, 1020, 970, 850 and 785 cm^{-1}; UV (MeOH): 274 and 292.5 nm; HRMS, $C_{18}H_{14}N_3F$ (M^+ 291.1161, calcd 291.1172); ^1H NMR (250 MHz, DMSO-d_6): δ 2.33 (3H, s, CH_3), 4.29 (2H, s, $-CH_2CN$), 6.96 (1H, s, H-4), 7.25 (2H, d, $J=7.98$ Hz, H-3′ and H-5′), 7.42–7.46 (2H, m, H-2′ and H-6′), 7.63–7.69 (2H, m, H-2″ and H-6″) and 7.77 (2H, d, $J=8.08$ Hz, H-3″ and H-5″); ^{13}C NMR (62.89 MHz, DMSO-d_6): δ 15.48 ($-CH_2CN$), 20.80 (CH_3), 104.72 (C-4), 116.09 ($-CH_2CN$), 116.46 (C-3″ and C-5″), 125.26 (C-3′ and C-5′), 126.95 (C-1′), 127.09 (C-2′ and C-6′), 129.31 (C-2″ and C-6″), 134.51(C-1″), 135.05 (C-3), 137.51 (C-4′), 150.88 (C-5) and 164.50 (C-4″); EI–MS, m/z (% rel. int.): 291 $[M]^+$ (10), 251(3), 130(6), 134(11), 91(43) and 95(100).

[3-(4-Fluorophenyl)-1-phenylpyrazol-5-yl]acetonitrile (8). White needles, mp 86–87 °C; IR (KBr): 3120, 2940, 2220, 1680, 1570, 1520, 1470, 1420, 1250, 1180, 1060, 1020, 940 and 755 cm^{-1}; UV (MeOH): 258 and 296 nm; ^1H NMR (300 MHz, $CDCl_3$): δ 3.79 (2H, s, $-CH_2CN$), 6.82 (1H, s, H-4), 7.11 (2H, m, H-3′ and H-5′), 7.48–7.56 (5H, m, H-2″, H-3″, H-4″, H-5″ and H-6″) and 7.85 (2H, m, H-2′ and H-6′); EI–MS, m/z (% rel. int.): 277 $[M]^+$ (100), 249(21), 155(22), 116(17), 95(26) and 77(94).

[3-(4-Methoxyphenyl)-1-phenylpyrazol-5-yl] acetonitrile (9). White solid, mp 102–103 °C; IR (KBr): 2240, 1620, 1510, 1440, 1260, 1180, 1040, 960, 840 and 770 cm^{-1}; UV (MeOH): 216 and 270 nm; HR-MS, $C_{18}H_{15}N_3O$ (M^+ 289.1201, calcd 289.1215); ^1H NMR (300 MHz, $CDCl_3$): δ 3.79 (2H, s, $-CH_2CN$), 3.86 (3H, s, $-OCH_3$), 6.80 (1H, s, H-4), 6.97 (2H, d, $J=8.7$ Hz, H-3′ and H-5′), 7.45–7.57 (5H, m, H-2″, H-3″, H-4″, H-5″ and H-6″) and 7.80 (2H, d, $J=8.7$ Hz, H-2′ and H-6′); EI–MS, m/z (% rel. int.): 289 $[M]^+$ (23), 288(100), 249(5), 146(5), 144(4) and 77(8).

[1-(4-Fluorophenyl)-3-phenylpyrazol-5-yl]acetonitrile (10). White solid, mp 126–127 °C; IR (KBr): 3080, 2900, 2260, 1615, 1560, 1515, 1470, 1375, 1220, 1095, 965, 840 and 700 cm^{-1}; UV (MeOH): 261 and 282.5 nm; HR-MS, $C_{17}H_{12}N_3F$ (M^+ 277.1041, calcd 277.1015); ^1H NMR (300 MHz, $CDCl_3$): δ 3.76 (2H, s, $-CH_2CN$), 6.86 (1H, s, H-4), 7.26 (2H, m, H-3″ and H-5″), 7.37–7.51 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′) and 7.18 (2H, d, $J=7.18$ Hz, H-2″ and H-6″); ^{13}C NMR (62.89 MHz, $CDCl_3$): δ 16.09 ($-CH_2CN$), 105.33 (C-4), 115.40 ($-CH_2CN$), 116.50 (C-3″ and C-5″), 125.73(C-4′), 125.70 (C-2′ and C-6′), 125.93 (C-2″ and C-6″), 127.43 (C-1″), 128.42 (C-1′), 132.85 (C-3′ and C-5′), 133.63 (C-3), 150.56 (C-5) and 163.78 (C-4″); EIMS, m/z (% rel. int.): 277 $[M]^+$ (29), 249(59), 216(22), 137(32), 109(42), 95(100), 84(42), 75(34) and 51(20).

[1,3-Di(4-bromophenyl)pyrazol-5-yl]acetonitrile (11). White crystals, mp 112–113 °C; IR (KBr): 2950, 2250, 1600, 1500, 1440, 1360, 1080, 1020, 960 and 840 cm^{-1}; UV (MeOH): 262 and 295 nm; HR-MS, $C_{17}H_{11}N_3Br_2$ (M^+ 414.9330, calcd 414.9320); ^1H NMR (250 MHz, $CDCl_3$): δ 3.77 (2H, s, $-CH_2CN$), 6.82 (1H, s, H-4), 7.35 (2H, d, $J=8.8$ Hz, H-2″ and H-6″), 7.54 (2H, d, $J=8.6$ Hz, H-2′ and H-6′), 7.67 (2H, d, $J=8.5$ Hz, H-3″ and H-5″) and 7.70 (2H, d, $J=8.5$ Hz, H-3′ and H-5′); ^{13}C NMR (62.89 MHz, $CDCl_3$): δ 16.18 ($-CH_2CN$), 105.67 (C-4), 115.25 ($-CH_2CN$), 122.50 (C-4″), 122.95 (C-4′), 126.62 (C-2′ and C-6′), 127.27 (C-2″ and C-6″), 131.06 (C-1′), 131.88 (C-3″ and C-5″), 132.90 (C-3′ and C-5′), 132.97 (C-1″), 137.39 (C-3) and 151.46 (C-5); EI–MS, m/z (% rel. int.): 415/419 $[M]^+/[M+4]^+$ (100.0/52.5), 387/389/391(5.5/10.0/6.0), 335/337(3.12/4.22), 309/311(2.4/2.4), 257(10.3), 207/208/209 (2.12/4.3/2.2), 194/196(3.2/3.3), 155/156/157 (6.57/2.97/4.9), 114/115(2.33/8.33) and 101/102(2.32/2.65) and 75(3.8).

[3-(4-Bromophenyl)-1-(4-fluorophenyl)pyrazol-5-yl]acetonitrile (12). White solid, mp 143–144 °C; IR (KBr): 2950, 2250, 1600, 1520, 1440, 1230, 1080, 1020, 970, 860 and 810 cm^{-1}; UV (MeOH): 263 and 292.5 nm; HR-MS, $C_{17}H_{11}N_3FBr$ (M^+ 355.0117, calcd 355.0120); ^1H NMR (250 MHz, $CDCl_3$): δ 3.74 (2H, s, $-CH_2CN$), 6.81 (1H, s, H-4), 7.25 (2H, d, $J=8.0$ Hz, H-2″ and H-6″), 7.45 (2H, d, $J=9.0$ Hz, H-3″ and H-5″), 7.54 (2H, d, $J=8.6$ Hz, H-2′ and H-6′) and 7.70 (2H, d, $J=8.6$ Hz, H-3′ and H-5′); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 16.09 ($-CH_2CN$), 105.26 (C-4), 115.30 ($-CH_2CN$), 116.93 (C-3″ and C-5″), 122.42 (C-4′), 127.27 (C-2′ and C-6′), 127.42 (C-2″ and C-6″), 131.17 (C-1′), 131.87 (C-3′ and C-5′), 133.14 (C-1″), 134.50 (C-3), 151.24 (C-5) and 164.61 (C-4″); EIMS, m/z (% rel. int.): 355/357 $[M]^+/[M+2]^+$ (100.0/97.4), 327/329(12.3/14.0), 315/317(4.3/4.4), 275/276(5.7/5.7), 249(6.4), 236(4.1), 194/196(2.0/2.2), 194(2.0), 178/179(4.4/1.5), 155(2.3), 147(2.5), 134/136(6.7/7.5), 111(2.7), 107(4.4) and 95(9.0).

[1-(4-Fluorophenyl)-3-(4-methoxyphenyl)pyrazol-5-yl]acetonitrile (13). White crystals, mp 144–145 °C; IR (KBr): 3050, 2900, 2250, 1620, 1520, 1460, 1370, 1310, 1250, 1180, 1030, 970, 840 and 780 cm^{-1}; UV (MeOH): 237, 264 and 288 nm; ^1H NMR (300 MHz, $CDCl_3$): δ 3.76 (2H, s, $-CH_2CN$), 3.86 (3H, s, $-OCH_3$), 6.79 (1H, s, H-4), 6.97 (2H, d, $J=8.7$ Hz, H-3′ and H-5′), 7.26 (2H, m, H-3″ and H-5″), 7.48 (2H, m, H-2″ and H-6″) and 7.78 (2H, d, $J=8.7$ Hz, H-2′ and H-6′); ^{13}C NMR (62.89 MHz, DMSO-d_6): δ 15.43 ($-CH_2CN$), 55.06 ($-OCH_3$), 104.42 (C-4), 116.02 (C-3″ and C-5″), 116.39 (C-3′ and C-5′), 116.86 ($-CH_2CN$), 124.82 (C-1′), 126.88 (C-2″ and C-6″), 127.02 (C-2′ and C-6′), 134.39 (C-1″), 135.09 (C-3), 150.73 (C-5), 159.27 (C-4′) and 163.35 (C-4″); EIMS, m/z (% rel. int.): 307 $[M]^+$ (25), 205(8), 169(3), 131(3), 146(1), 134(1), 95(1) and 18(100).

[3-(4-Chlorophenyl)-1-(4-fluorophenyl)pyrazol-5-yl]acetonitrile (14). White solid, mp 130–131 °C; IR (KBr): 3080, 2280, 1610, 1520, 1425, 1370, 1240, 1100, 1020, 850 and 740 cm^{-1}; UV (MeOH): 274 and 295 nm; HR-MS, $C_{17}H_{11}N_3FCl$ (M^+ 311.0627, calcd 311.0629); ^1H NMR (60 MHz, $CDCl_3$): δ 3.78 (2H, s, $-CH_2CN$), 6.88

(1H, s, H-4), 7.10–7.35 (6H, m, H-2′, H-2″, H-3′, H-5′, H-6′ and H-6″) and 7.47 (2H, d, $J=8.0$ Hz, H-3″, H-5″); ^{13}C NMR (62.8 MHz, DMSO-d_6): δ 15.45 (–CH_2CN), 105.00 (C-4), 116.08 (C-3″ and C-5″), 116.45 (C-2″ and C-6″), 116.75 (–CH_2*C*N), 127.02 (C-3′ and C-5′), 127.15 (C-1′), 128.73 (C-2′ and C-6′), 131.08 (C-1″), 132.66 (C-3), 134.89 (C-4′), 149.70 (C-5) and 163.51 (C-4″); EI–MS, *m/z* (% rel. int.): 311/313 $[M]^+/[M+2]^+$ (100.0/34.0), 155.5/156.5(3.0/1.0), 271/273(7.0/2.0), 150/152(4.0/1.0), 134(8.0), 111/113(4.0/1.0) and 95(16.0).

[1,3-Di(4-fluorophenyl)pyrazol-5-yl]acetonitrile (15). White solid, mp 134–136 °C; IR (KBr): 3080, 3000, 2260, 1615, 1520, 1450, 1360, 1230, 1150, 1100, 960 and 840 cm^{-1}; UV (MeOH): 225 and 287 nm; HR-MS, $C_{17}H_{11}N_3F_2$ (M^+ 295.0930, calcd 295.0921); ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.74 (2H, s, –CH_2CN), 6.79 (1H, s, H-4), 7.12 (2H, m, H-3″ and H-5″), 7.25 (2H, m, H-3′ and H-5′), 7.45 (2H, d, $J=9.1$ Hz, H-2″ and H-6″) and 7.81 (2H, d, $J=8.9$ Hz, H-2′ and H-6′); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 16.05 (–*C*H_2CN), 105.11 (C-4), 115.35 (–CH_2*C*N), 115.85 (C-3″ and C-5″), 116.88 (C-3′ and C-5′), 127.26 (C-2″ and C-6″), 127.40 (C-2′ and C-6′), 128.41 (C-1′), 133.02 (C-1″), 134.55 (C-3), 151.42 (C-5), 160.95 and 164.89 (C-4″ and C-4′); EIMS, *m/z* (% rel. int.): 295 $[M]^+$ (100), 294(27), 267(24), 255(7), 216(2), 173(5), 148(4), 138(5), 96(9) and 75(3).

[1-(4-Chlorophenyl)-3-(4-methoxyphenyl)pyrazol-5-yl]acetonitrile (16). White fluffy solid, mp 142–143 °C; IR (KBr): 2980, 2900, 2280, 1620, 1535, 1500, 1450, 1370, 1250, 1180, 1090, 970 and 840 cm^{-1}; UV (MeOH): 236, 265 and 291 nm; HR-MS, $C_{18}H_{14}N_3OCl$ (M^+ 323.0834, calcd 323.0825); ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.78 (2H, s, CH_2CN), 3.86 (3H, s, OCH_3), 6.80 (1H, s, C-4H), 6.96 (2H, d, $J=8.3$ Hz, C-3′H and C-5′H), 7.44 (2H, d, $J=8.4$ Hz, C-2″H and C-6″H), 7.52 (2H, d, $J=8.3$ Hz, C-2′H and C-6′H) and 7.78 (2H, d, $J=8.4$ Hz, C-3″H and C-5″H); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 15.51 (*C*H_2CN), 55.06 (OCH_3), 104.35 (C-4), 114.10 (C-3″ and C-5″), 116.84 (CH_2*C*N), 124.68 (C-3′ and C-5′), 124.80 (C-1′), 126.68 (C-2″ and C-6″), 129.35 (C-2′ and C-6′), 132.53 (C-4″), 134.41 (C-5), 137.51 (C-4′), 150.98 (C-1″) and 159.33 (C-3); EI–MS, *m/z* (% rel. int.): 325 $[M+2]^+$ (33), 323 $[M]^+$ (100), 308(14), 280(4), 205(2), 111(9) and 90(2).

3-Amino-6-(4-fluorophenyl)-2-phenyl-4-oxo-4*H*-pyrano[4,3-*c*] pyrazole (19). White solid, mp 255–257 °C; IR (KBr): 3470, 3360, 3100, 1735, 1645, 1570, 1510, 1370, 1230, 1170, 1040, 920 and 855 cm^{-1}; UV (MeOH): 250, 275 and 295 nm; HR-MS, $C_{18}H_{12}N_3O_2F$ (M^+ 321.0932, calcd 321.0914); ^{1}H NMR (60 MHz, $CDCl_3$ + TFA): δ 6.48 (1H, s, H-7), 7.30 (7H, brm, H-2″, H-3′, H-3″, H-4″, H-5′, H-5″ and H-6″) and 7.68 (2H, m, H-2′ and H-6′); ^{13}C NMR (62.89 MHz, DMSO-d_6): δ 89.71 (C-7), 95.21 (C-9), 115.57 (C-3′ and C-5′), 115.92 (C-2′ and C-6′), 124.02 (C-2″ and C-6″), 127.88 (C-1′), 129.44 (C-3″, C-4″ and C-5″), 137.44 (C-6), 148.26 (C-1″), 154.37 (C-8), 158.19 (C-3), 160.83 (C-4) and 164.77 (C-4′); EI–MS, *m/z* (% rel. int.): 321 $[M]^+$ (100.0), 293(31.9), 264(22.8), 226(7.4), 123(17.0), 119(7.4), 95(20.9) and 77(74.8).

3-Amino-6-(3,4-methylenedioxyphenyl)-2-phenyl-4-oxo-4*H*-pyrano[4,3-*c*]pyrazole (20). Brown solid, mp 259–262 °C; IR (KBr): 3392, 3282, 2901, 2791, 1722, 1637, 1494, 1443, 1355, 1271, 1037 and 950 cm^{-1}; UV (MeOH): 235, 334 and 345 nm; HR-MS, $C_{19}H_{13}N_3O_4$ (M^+ 347.0898, calcd 347.0906); ^{1}H NMR (60 MHz, $CDCl_3$ + TFA): δ 6.14 (2H, s, –OCH_2O-), 6.80 (1H, s, H-2′), 7.04 (1H, s, H-7), 7.36–7.50 (2H, m, H-5′ and H-6′) and 7.70 (5H, brm, H-2″, H-3″, H-4″, H-5″ and H-6″); EI–MS, *m/z* (% rel. int.): 347 $[M]^+$ (100.0), 319(34.2), 290(16.7), 226(5.4), 149(12.1), 121(4.2), 119(8.1) and 77(22.1).

3-Amino-2-(4-fluorophenyl)-6-(4-methylphenyl)-4-oxo-4*H*-pyrano [4,3-*c*]pyrazole (21). White solid, mp > 300 °C; IR (KBr): 3350, 2750, 1730, 1640, 1450, 1380, 1165, 1050, 840 and 740 cm^{-1}; UV (MeOH): 250, 260 and 275 nm; ^{1}H NMR (90 MHz, $CDCl_3$ + TFA): δ 2.38 (3H, s, CH_3), 7.03 (1H, s, H-7), 7.15–7.42 (4H, brm, H-3′, H-3″, H-5′ and H-5″), 7.50 (2H, d, $J=8.0$ Hz, H-2″ and H-6″) and 7.72 (2H, d, $J=8.3$ Hz, H-2′ and H-6′); ^{13}C NMR (62.89 MHz, DMSO-d_6): δ 20.79 (CH_3), 89.59 (C-7), 94.43 (C-9), 124.93 (C-3′ and C-5′), 126.68 (C-3″ and C-5″), 126.83 (C-2″ and C-6″), 129.34 (C-2′ and C-6′), 129.48 (C-6), 133.82 (C-1′), 139.46 (C-4′), 148.47 (C-1″), 155.47 (C-8), 158.32 (C-3), 163.13 (C-4″) and 173.90 (C-4); EI–MS, *m/z* (% rel. int.): 335 $[M]^+$ (100.0), 307(17.0), 278(9.0), 244(4.0), 167.5(3.0), 153.5(4.0), 137(5.0), 119(10.0), 95(12.0) and 91(9.0).

3-Amino-6-(4-chlorophenyl)-2-(4-fluorophenyl)-4-oxo-4*H*-pyrano[4,3-*c*]pyrazole (22). Pale white solid, mp 242 °C; IR (KBr): 3345, 3122, 2929, 1720, 1637, 1509, 1404, 1254, 1095, 1037 and 809 cm^{-1}; UV (MeOH): 250, 300 and 312 nm; ^{1}H NMR (60 MHz, $CDCl_3$ + TFA): δ 7.16 (1H, s, H-7), 7.40 (4H, m, H-3′, H-3″, H-5′ and H-5″) and 7.70 (4H, m, H-2′, H-2″, H-6′ and H-6″); ^{13}C NMR (62.8 MHz, DMSO-d_6): δ 89.57 (C-7), 95.76 (C-9), 116.03 (C-3″ and C-5″), 116.40 (C-2″ and C-6″), 126.71 (C-3′ and C-5′), 126.82 (C-1′), 127.92 (C-2′ and C-6′), 131.05 (C-6), 134.22 (C-4′), 148.51 (C-1″), 154.11 (C-8), 157.96 (C-3), 159.24 (C-4″) and 163.14 (C-4); EI–MS, *m/z* (% rel. int.): 355/357 $[M]^+/[M+2]^+$ (11.0/4.0), 327/329(1.0/1.0), 298/300(1.0/1.0), 274/277(11.0/4.0), 246(2.0), 203(1.0), 163(2.0), 139/141(16.0/5.0), 111/115(5.0/2.0), 95(2.0), 73(11.0), 60(16.0), 43(10.0) and 18(100).

3-Amino-2,6-di(4-fluorophenyl)-4-oxo-4*H*-pyrano[4,3-*c*]pyrazole (23). White solid, mp > 300 °C, IR (KBr): 3440, 3080, 2900, 1725, 1645, 1515, 1420, 1225, 1165, 1100, 1050, 925 and 815 cm^{-1}; UV (MeOH): 286 and 296 nm; HR-MS, $C_{18}H_{11}N_3O_2F_2$ (M^+ 339.0821, calcd 339.0819); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.08 (1H, s, H-7), 7.17 (2H, d, $J=8.5$ Hz, H-3″ and H-5″), 7.34 (2H, d, $J=8.5$ Hz, H-3′ and H-5′), 7.76 (2H, d, $J=8.5$ Hz, H-2′ and H-6′) and 7.87 (2H, d, $J=8.8$ Hz, H-2″ and H-6″); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 91.04 (C-9), 92.22 (C-7), 117.96 (C-3″ and C-5″), 118.33 (C-3′ and C-5′), 126.32 (C-2″ and C-6″), 128.40 (C-2′ and C-6′), 128.84 (C-6), 144.63 (C-1″), 148.09 (C-8), 149.21 (C-1′), 159.69 (C-3), 160.21 and 161.57 (C-4′ and C-4″) and 161.77 (C-4); EI–MS, *m/z* (% rel. int.): 339 $[M]^+$ (100), 311(29),

282(15), 244(4.0), 188(3.2), 169(3.1), 155(2.4), 137(4.1), 123(9.7), 111(4.12), 109(2.8), 95(11.9) and 75(2.2).

3-Amino-2-(4-chlorophenyl)-6-(4-fluorophenyl)-4-oxo-4*H*-pyrano[4,3-*c*]pyrazole (24). Pale yellow solid, mp >300 °C; IR (KBr): 3440, 3340, 2940, 1710, 1640, 1425, 1400, 1310, 1260, 1100, 1040, 920 and 840 cm^{-1}; UV (MeOH): 250, 300 and 312 nm; HR-MS, $C_{18}H_{11}N_3O_2FCl$ (M^+ 355.0511, calcd 355.0524); ^1H NMR (250 MHz, DMSO-d_6): δ 6.90 (2H, brs, $-NH_2$), 7.13 (1H, s, H-7), 7.35 (2H, m, H-3′ and H-5′), 7.61 (4H, bs, H-2″, H-3″, H-5″ and H-6″) and 7.81 (2H, d, J = 8.4 Hz, H-2′ and H-6′); EI–MS, m/z (% rel. int.): 357 $[M+2]^+$ (16.7), 355 $[M]^+$ (46.2), 327(14.5), 153/154(9.0/18.5), 123(16.9), 113(7.9) and 95(12.6).

3-Amino-2-(4-fluorophenyl)-6-(4-methoxyphenyl)-4-oxo-4*H*-pyrano [4,3-*c*]pyrazole (25). Pale yellow solid, mp 178–180 °C; IR (KBr): 3357, 2928, 1719, 1636, 1509, 1256, 1180, 1033 and 839 cm^{-1}; UV (MeOH): 254, 291 and 303 nm; ^1H NMR (250 MHz, DMSO-d_6): δ 3.82 (3H, s, $-OCH_3$), 6.77 (1H, s, H-7), 7.04 (2H, d, J = 8.96 Hz, H-3′ and H-5′), 7.40 (2H, m, H-3″ and H-5″), 7.61 (2H, m, H-2″ and H-6″) and 7.82 (2H, d, J = 8.92 Hz, H-2′ and H-6′); ^{13}C NMR (62.8 MHz, DMSO-d_6): δ 55.22 ($-OCH_3$), 89.47 (C-7), 93.41 (C-9), 116.02 (C-3″ and C-5″), 116.39 (C-2″ and C-6″), 126.58 (C-3′ and C-5′), 126.72 (C-2′ and C-6′), 130.74 (C-1′), 133.82 (C-6), 148.41 (C-8), 149.12 (C-1″), 155.42 (C-3), 158.35 (C-4′), 160.42 (C-4) and 163.07 (C-4″); EI–MS, m/z (% rel. int.): 351 $[M]^+$ (100.0), 323(13.0), 294(12.0), 244(3.0), 175.5(2.0), 161.5(5.0), 137(7.0), 135(17.0) and 95(22.0).

3-Amino-2-(4-chlorophenyl)-6-(4-methoxyphenyl)-4-oxo-4*H*-pyrano[4,3-*c*] pyrazole (26). Buff coloured solid, mp >300 °C; IR (KBr): 3400, 1740, 1640, 1530, 1470, 1380, 1260, 1170, 1030 and 830 cm^{-1}; UV (MeOH): 242, 302 and 390 nm; HR-MS, $C_{19}H_{14}N_3O_3Cl$ (M^+ 367.0534, calcd 367.0724); ^1H NMR (250 MHz, DMSO-d_6): δ 3.82 (3H, s, OCH_3), 6.89 (2H, brs, NH_2), 7.04 (3H, m, C-3′H, C-5′H and C-7H), 7.59–7.62 (4H, m, C-2″H, C-6″H, C-2′H and C-6′H) and 7.82 (2H, d, J = 8.4 Hz, C-3″H and C-5″H); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 55.36 (OCH_3), 89.70 (C-9), 93.46 (C-7), 114.30 (C-3″ and C-5″), 124.66 (C-3′ and C-5′), 126.01 (C-2″ and C-6″), 126.75 (C-2′ and C-6′), 129.52 (C-6), 132.25(C-1″), 136.43 (C-4″), 148.55 (C-8), 149.50 (C-1′), 155.61 (C-4′), 158.48 (C-3) and 160.55 (C-4); EI–MS, m/z (% rel. int.): 369 $[M+2]^+$ (33), 367 $[M]^+$ (100), 341(3), 339(10), 310(8), 272(2), 185(2), 135(17), 127(6), 111(13) and 77(10).

(*Z*)-2-(1,3-Diphenylpyrazol-5-yl)-3-(3,4-difluorophenyl) acrylonitrile (38). White solid, mp 154–155 °C; IR (KBr): 3447, 2924, 2363, 2215, 1598, 1502, 1429, 1281, 1152, 903, 816, 770, 690 and 526 cm^{-1}; UV (MeOH): 207, 261 and 310 nm; HR-MS, $C_{24}H_{15}N_3F_2$ (M^+ 383.1233, calcd 383.1234); ^1H NMR (250 MHz, $CDCl_3$); δ 6.96 (1H, s, H-3), 7.04 (1H, s, H-4′), 7.22–7.26 (1H, m, H-2‴), 7.35–7.48 (5H, m, H-3″, H-4″, H-4⁗, H-5″, H-5‴), 7.51–7.54 (4H, m, H-2″, H-3⁗, H-5⁗, H-6″), 7.56–7.65 (1H, m, H-6‴), 7.88 (2H, m, H-2⁗, H-6⁗); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 101.79 (CN), 106.37 (C-4′), 115.64 (C-2), 118.21 (C-5‴), 125.54, 125.79 (C-2″, C-3″, C-5″ and C-6″), 126.31 (C-2‴), 128.47, 128.88 (C-4⁗, C-4″), 128.75, 129.70 (C-2⁗, C-3⁗, C-5⁗, C-6⁗), 129.76, 129.80 (C-6‴, C-3), 132.11 (C-1″), 137.79, 139.31 (C-1⁗, C-1‴), 143.76, 149.74, 152.49, 154.02 (C-3′, C-3‴, C-4‴ and C-5′); EI–MS, (rel. int.) m/z (%): 383 $[M]^+$ (100), 382(4), 357(10), 279(5), 271(20), 243(7), 180(8), 167(7) and 77(14).

(*Z*)-2-[3-(4-Chlorophenyl)-1-phenylpyrazol-5-yl]-3-(3,4-difluorophenyl) acrylonitrile (39). White solid, mp 137–138 °C; IR (KBr): 3436, 2925, 2215, 1596, 1522, 1496, 1434, 1285, 1161, 1088, 925, 818 cm^{-1}; UV (MeOH): 211, 263 and 307 nm; HR-MS, $C_{24}H_{14}N_3F_2Cl$ (M^+ 417.0824, calcd 417.0844); ^1H NMR (250 MHz, $CDCl_3$); δ 6.93 (1H, s, H-3), 7.03 (1H, s, H-4′), 7.20–7.27 (1H, m, H-2‴), 7.40 (2H, d, J = 8.7 Hz, H-3″ and H-5″), 7.47–7.54 (6H, m, H-2″, H-3⁗, H-4⁗, H-5‴, H-5⁗ and H-6″), 7.57–7.66 (1H, m, H-6‴), 7.82 (2H, m, H-2⁗ and H-6⁗); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 101.61 (CN), 106.26 (C-4), 115.59 (C-2), 118.14 (C-5‴), 125.52 (C-2⁗, C-6⁗), 126.42 (C-2‴), 127.04 (C-3″ and C-5″), 128.96 (C-3⁗ and C-5⁗), 129.03 (C-4⁗), 129.56 (C-2″ and C-6″), 129.63, 129.73 (C-6‴ and C-3), 130.07 (C-4″), 134.29 (C-1″), 138.00, 139.19 (C-1‴ and C-1⁗), 143.95 (C-3′), 149.80 (C-3‴), 152.52 (C-5′) and 153.88 (C-4‴); EI–MS, (rel. int.) m/z (%): 419 ($[M+2]^+$, 85), 417 ($[M]^+$, 100), 416(35), 354(4), 306(28), 304(76), 242(9), 167(9) and 77(14).

(*Z*)-2-[3-(4-Bromophenyl)-1-phenylpyrazol-5-yl]-3-(3,4-difluorophenyl) acrylonitrile (40). White solid, mp 145–146 °C; IR (KBr): 3433, 2925, 2215, 2064, 1595, 1521, 1496, 1289, 1169, 1071, 925, 816, 758, 695 and 500 cm^{-1}; UV (MeOH): 208 and 270 nm; HR-MS, $C_{24}H_{14}N_3F_2Br$ (M^+ 461.0339, calcd 461.0339); ^1H NMR (250 MHz, $CDCl_3$): δ 6.94 (1H, s, H-3), 7.02 (1H, s, H-4′), 7.19–7.26 (1H, m, H-2‴), 7.44–7.57 (8H, m, H-2″, H-3″, H-3⁗, H-4⁗, H-5⁗, H-5″, H-5‴ and H-6″), 7.59–7.65 (1H, m, H-6‴) and 7.54 (2H, m, H-2⁗ and H-6⁗); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 101.56 (CN), 106.22 (C-4′), 115.56 (C-2), 118.23 (C-5‴), 122.47 (C-4″), 125.48 (C-2⁗ and C-6⁗), 126.41 (C-2‴), 127.29 (C-2″ and C-6″), 129.02 (C-4⁗), 129.60 (C-3⁗ and C-5⁗), 129.01, 129.70 (C-6‴ and C-3), 131.09 (C-1″), 131.87 (C-3″ and C-5″), 138.00, 139.15 (C-1‴ and C-1⁗), 143.94 (C-3′), 151.14 (C-5′), 149.77 and 153.85 (C-3‴ and C-4‴); EI–MS, (rel. int.) m/z (%): 463 ($[M+2]^+$, 96), 461 ($[M]^+$, 100), 460(30), 435(9), 289(6), 269(23), 242(8), 167(13) and 77(17).

(*Z*)-2-[3-(4-Methylphenyl)-1-phenylpyrazol-5-yl]-3-(3,4-difluorophenyl) acrylonitrile (41). White solid mp 143-144 °C; IR (KBr): 3435, 2923, 2215, 1597, 1521, 1499, 1434, 1286, 1169, 1061, 921, 805 cm^{-1}; UV (MeOH): 207, 267 and 307 nm; HR-MS, $C_{25}H_{17}N_3F_2$ (M^+ 397.1379, calcd 397.1391); ^1H NMR (250 MHz, $CDCl_3$): δ 2.38 (3H, s, $-CH_3$), 6.92 (1H, s, H-3), 7.02 (1H, s, H-4′), 7.15–7.18 (1H, m, H-2‴), 7.23 (2H, d, J = 7.98 Hz, H-3″ and H-5″), 7.42–7.47 (2H, m, H-4⁗, H-5‴), 7.40–7.53 (4H, m, H-2″, H-3⁗, H-5⁗ and H-6″), 7.56–7.64 (1H, m, H-6‴), 7.76 (2H, m, H-2⁗ and H-6⁗); ^{13}C

NMR (62.8 MHz, $CDCl_3$): δ 21.27 (–CH_3), 101.83 (CN), 106.21 (C-4′), 115.66 (C-2), 118.08 (C-5‴), 125.52, 125.68 (C-2⁗, C-2″, C-6⁗ and C-6″), 126.35 (C-2‴), 128.78 (C-4⁗), 129.29 (C-4″), 129.43, 129.53, 129.66 (C-3‴, C-3′, C-5‴, C-5′ and C-β), 129.76 (C-6‴), 137.68, 138.34, 139.35 (C-1″, C-1‴ and C-1⁗), 143.64 (C-3′), 149.69 (C-5′), 152.30 and 152.47 (C-3‴ and C-4‴); EI–MS, (rel. int.) *m/z* (%): 397 ([M^+], 100), 396(36), 371(8), 284(65), 257(3), 194(8), 167(4) and 77(8).

(*Z*)-2-[3-(4-Methoxyphenyl)-1-phenylpyrazol-5-yl]-3-(3,4-difluorophenyl) acrylonitrile (42). Light yellow solid, mp 135–137 °C; IR (KBr): 3436, 2967, 2215, 1612, 1522, 1438, 1291, 1258, 1029, 840, 761, 696 and 522 cm^{-1}; UV (MeOH): 271 and 209 nm; HR-MS, $C_{25}H_{17}N_3OF_2$ (M^+ 413.1325, calcd 413.1340); ^1H NMR (250 MHz, $CDCl_3$): δ 3.84 (3H, s, –OCH_3), 6.88 (1H, s, H-3), 6.95 (2H, d, J = 8.9 Hz, H-3″ and H-5″), 7.04 (1H, s, H-4′), 7.16–7.25 (1H, m, H-2‴), 7.46–7.53 (6H, m, H-2″, H-3⁗, H-4⁗, H-5‴, H-5⁗ and H-6″), 7.56–7.63 (1H, m, H-6‴) and 7.81 (2H, m, H-2⁗ and H-6⁗); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 55.28 (OCH_3), 101.82 (CN), 105.94 (C-4′), 114.13 (C-3″ and C-5″), 115.67 (C-2), 118.06 (C-5‴), 124.83 (C-1″), 126.49 (C-2⁗ and C-6⁗), 126.35 (C-2‴), 127.07 (C-3⁗ and C-5⁗), 128.75 (C-4⁗), 129.66 (C-3), 129.53 (C-2″ and C-6″), 129.76 (C-6‴), 137.67, 139.34 (C-1‴ and C-1⁗), 143.62 (C-3′), 149.68, 153.75 (C-3‴ and C-4‴), 152.07 (C-5′) and 159.91 (C-4″); EI–MS, *m/z* (rel. int.): 413 $[M]^+$ (100), 398(9), 370(4), 300(30), 210(7), 150(3) and 77(6).

[3-(3,4-Methylenedioxyphenyl)isoxazol-5-yl] acetonitrile (46). White solid, mp 161 °C; IR (nujol): 2240, 2070, 1599, 1498, 1382, 1265, 1227, 1147, 942, 885 and 810 cm^{-1}; UV (MeOH): 295, 260 and 215 nm; ^1H NMR (250 MHz, $CDCl_3$): δ 2.24 (2H, s, CH_2CN), 5.98 (2H, s, OCH_2O), 6.70 (1H, d, J = 8.1 Hz, H-5′), 6.82 (1H, s, H-4), 7.00 (1H, d, J = 2.0 Hz, H-2′) and 7.10 (1H, m, H-6′); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 12.20 (–CH_2CN), 101.29 (–OCH_2O–), 106.23 (C-2′), 108.05 (C-5′), 109.50 (CN), 112.98 (C-4), 120.36 (C-6′), 130.75 (C-1′), 147.9 (C-4′), 148.56 (C-3′), 150.10 (C-5) and 155.56 (C-3); EI–MS, *m/z* (rel. int.%): 228 [M^+].

3-(3,4-Methylenedioxyphenyl)-5-(methylthio)-4,5-dihydro-isoxazol-5-yl] acetonitrile (48). White solid, mp 82 °C; IR (KBr): 2264, 1608, 1532, 1260, 1243, 1133, 1045, 935 and 912 cm^{-1}; UV (MeOH): 305, 270 and 219 nm; HR-MS, $C_{13}H_{12}N_2O_3S$ (M^+ 276.0587, calcd 276.0569); ^1H NMR (250 MHz, $CDCl_3$): δ 2.25 (3H, s, SCH_3), 3.20 (2H, s, CH_2CN), 3.46 (1H, d, J = 17.6 Hz, H-4a), 3.70 (1H, d, J = 17.5 Hz, H-4b), 6.00 (2H, s, OCH_2O), 6.70 (2H, m, H-2′ and H-6′) and 7.02 (1H, d, J = 8.0 Hz, H-5′); ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.67 (CH_2CN), 16.63 (SCH_3), 28.74 (C-4), 46.73 (C-5), 101.00 (OCH_2O), 104.00 (CN), 106.79, 108.26, 108.62, 121.85 and 122.12 (C-3, C-1′, C-2′, C-5′ and C-6′), and 148.23 and 149.92 (C-3′ and C-4′); EI–MS, *m/z* (rel. int.): 276 $[M]^+$ (53), 229(100), 228(83), 201(17), 188(51), 171(21), 161(81), 160(36), 146(20), 121(22), 63(18) and 44(11).

3-Amino-6-(4-fluorophenyl)pyrano[4,3-*c*]isoxazole-4-one (51). White solid, mp 240–242 °C; IR (Nujol): 3365, 1720, 1600 and 1550 cm^{-1}; UV (MeOH): 310, 271, 259, 254 and 228 nm; ^1H NMR (250 MHz, $CDCl_3$ + TFA): δ 6.50 (1H, s, H-7), 7.15 (2H, m, H-3′ and H-5′), 7.80 (2H, m, H-2′ and H-6′) and 9.20 (2H, brs, NH_2); ^{13}C NMR (62.8 MHz, DMSO-d_6): δ 98.29, 127.31, 128.47, 128.55, 128.98, 129.28, 129.35, 136.05, 156.96, 161.95, 162.80 and 169.68; EI–MS, *m/z* (rel. int.%): 246 $[M]^+$ (5), 230(10), 201(5), 187(8), 163(7), 135(6), 123(100), 107(12), 95(56), 75(24) and 42(24).

[3-(4-Chlorophenyl)isoxazol-5-yl]-2*H*-1-benzopyran-2-one (52). White solid, mp 242–244 °C; IR (KBr): 1740, 1675, 1608, 1440, 1275, 1218, 1195, 1101, 817 and 752 cm^{-1}; UV (MeOH): 350, 320, 305 and 245 nm; ^1H NMR (300 MHz, $CDCl_3$ + traces of TFA): δ 7.30 (1H, s, H-4′), 7.45–7.47 (2H, m, H-7 and H-8), 7.70–7.90 (4H, m, H-3″, H-5″, H-5 and H-6), 7.93 (2H, d, J = 8.5 Hz, H-2″ and H-6″) and 8.73 (1H, s, H-4); ^{13}C NMR (75 MHz, $CDCl_3$ + 1 drop of TFA): δ 103.07, 117.39, 128.27, 128.55, 129.10, 129.38, 129.52, 130.27, 133.68, 137.31, 146.74, 151.01, 159.60, 160.15, 162.78 and 163.09; EI–MS, *m/z* (rel. int.): 323 $[M]^+$ (20), 322(60), 294(10), 206(11), 198(30), 186(25), 173(100), 150(10), 129(25), 107(72) and 75(18).

3-[3-(4-Methylphenyl)isoxazol-5-yl]-2*H*-1-benzopyran-2-one (53). White solid, mp 212 °C; IR (Nujol): 1740, 1667, 1608, 1282, 1225, 952 and 882 cm^{-1}; UV (MeOH): 355, 345 and 325 nm; ^1H NMR (250 MHz, $CDCl_3$): δ 2.40 (3H, s, CH_3), 7.15–7.21 (2H, m, H-7 and H-8), 7.28 (2H, d, J = 8.0 Hz, H-3″ and H-5″), 7.39–7.49 (2H, m, H-5 and H-6), 7.59 (1H, s, H-4′), 7.78 (2H, d, J = 8.0 Hz, H-2″ and H-6″) and 8.01 (1H, s, H-4); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 21.41 (CH_3), 104.07 (C-4′), 115.40 (C-8), 116.50 (C-10), 118.83 (C-4″), 123.90 (C-7), 126.16 (C-3), 126.80 (C-3″ and C-5″), 128.69(C-1″), 129.54 (C-2″ and C-6″), 131.82 (C-6), 134.08 (C-5), 140.13 (C-4), 153.35, 163.11 and 163.25 (C-2, C-3′, C-5′ and C-9); EI–MS, *m/z* (rel. int.): 303 [M^+] (8), 302(35), 274(3), 211(4), 186(13), 185(100), 158(5), 130(8), 102(4), 91(10) and 65(5).

3-[3-(3,4-Methylenedioxyphenyl)isoxazol-5-yl]-2*H*-1-benzopyran-2-one (54). White solid, mp 235–237 °C; IR (Nujol): 1720, 1663, 1604, 1462, 1282, 1251, 1213, 1048, 937 and 811 cm^{-1}; UV (MeOH): 345, 310 and 305 nm; ^1H NMR (250 MHz, $CDCl_3$ + TFA): δ 6.06 (2H, s, –OCH_2O–), 6.92 (1H, d, J = 7.9 Hz, H-5″), 7.34–7.48 (5H, m, H-4′, H-2″, H-6″, H-7 and H-8), 7.66–7.74 (2H, m, H-5 and H-6) and 8.55 (1H, s, H-4); ^{13}C NMR (62.8 MHz, $CDCl_3$–TFA): δ 101.73 (C-4′), 103.98 (–OCH_2O–), 106.92 (C-2″), 108.96 (C-5″), 116.75 (C-3), 116.97 (C-8), 117.59 (C-1″), 118.44 (C-10), 121.29, 122.04 (C-6″ and C-7), 125.68 (C-6), 129.34 (C-5), 133.84 (C-4), 159.31, 159.99, 160.64, 162.88, 163.12 and 163.44 (C-2, C-3′, C-3″, C-4″, C-5′ and C-9); EI–MS, *m/z* (rel. int.%): 333 $[M]^+$ (100), 332(40), 305(33), 277(5), 249(6), 210(6), 188(15), 185(14), 173(54), 163(17), 160(18), 146(12), 130(7), 101(8) and 89(6).

7,8-Dibutanoyloxy-4-methyl-2*H*-1-benzopyran-2-one (58). White solid mp 99–100 °C; IR (KBr): 3084, 2972, 2935, 2877, 2363, 1764, 1738, 1612, 1575, 1443, 1372, 1268,

1235, 1137, 1096, 1051, 953, 869, 825, 745 and 589 cm^{-1}; UV (MeOH): 308, 277 and 211 nm; HR-MS, $C_{18}H_{20}O_6$ (M^+ 332.1279, calcd 332.1260); ^1H NMR (300 MHz, $CDCl_3$): δ 1.10 (6H, m, 2×$CH_2CH_2CH_3$), 1.85 (4H, m, 2×$CH_2CH_2CH_3$), 2.47 (3H, s, C-4CH_3), 2.60 (2H, t, CH_2–C=O), 2.69 (2H, t, CH_2–C=O), 6.30 (1H, s, H-3), 7.19 (1H, d, J=8.7 Hz, H-6) and 7.53(1H, d, J=8.7 Hz, H-5); ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.55 (2 × $CH_2CH_2CH_3$), 18.29 and 18.37 (2·$CH_2CH_2CH_3$), 18.76 (C-4CH_3), 35.45 and 35.77 (2 × $CH_3CH_2CH_2$–C=O), 114.69 (C-10), 118.54 (C-6), 118.72 (C-3), 121.48 (C-5), 130.40 (C-4), 145.27 and 146.69 (C-7 and C-8), 151.87 (C-9), 159.18 (C-2), and 170.11 and 170.47 (2×C=O); CI-MS, m/z (rel. int.): 333 $[M+1]^+$ (100).

7-Butanoyloxy-4-methyl-2*H*-1-benzopyran-2-one (59). White solid, mp 92–93 °C; IR (KBr): 2977, 2364, 1754, 1731, 1616, 1571, 1420, 1386, 1371, 1262, 1148, 1091, 1061, 983, 926, 873, 857, 817, 741, 614 and 454 cm^{-1}; UV (MeOH): 312, 274 and 210 nm; HR-MS, $C_{14}H_{14}O_4$ (M^+ 246.0879, calcd 246.0892); ^1H NMR (300 MHz, $CDCl_3$): δ 1.62 (3H, t, $CH_2CH_2CH_3$), 1.85 (2H, m, $CH_2CH_2CH_3$), 2.48 (3H, s, C-4CH_3), 2.64 (2H, t, $CH_3CH_2CH_2$C=O), 6.28 (1H, s, H-3), 7.11 (2H, m, H-6 and H-8) and 7.65 (1H, d, J=8.5 H_z, H-5); ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.53 ($CH_2CH_2CH_3$), 18.24 ($CH_2CH_2CH_3$), 18.68 (C-4CH_3), 36.06 ($CH_3CH_2CH_2$C=O), 110.38 (C-8), 114.37 (C-10), 117.67 (C-6), 118.07 (C-3), 125.27 (C-5), 151.89 (C-4), 153.08 (C-9), 154.07 (C-7), 160.48 (C-2) and 171.41 (C=O); EI–MS, m/z (rel. int.): 246 $[M]^+$ (15), 177(25), 176(100), 148(45), 147(12), 91(17), 86(38), 84(64), 71(88), 65(12), 51(36) and 49(83).

5,7-Dibutanoyloxy-4-methyl-2*H*-1-benzopyran-2-one (60). White solid, mp 79–80 °C; IR (KBr): 3446, 3080, 2932, 1760, 1724, 1617, 1453, 1419, 1361, 1295, 1141, 1060, 1003, 925, 855, 747, 701 and 533 cm^{-1}; UV (MeOH): 284, 279 and 208 nm; HR-MS, $C_{18}H_{20}O_6$ (M^+ 332.1252, calcd 332.1260); ^1H NMR (300 MHz, $CDCl_3$): δ 1.10 (6H, m, 2×$CH_2CH_2CH_3$), 1.82 (4H, m, 2×$CH_2CH_2CH_3$), 2.54 (3H, s, C-4CH_3), 2.64 (4H, m, 2×$CH_3CH_2CH_2$–C=O), 6.20 (1H, s, H-3), 6.84 (1H, d, J=2.0 Hz, H-6) and 7.12 (1H, d, J=2.0 Hz, H-8); ^{13}C NMR (75 MHz, $CDCl_3$); δ 13.52 and 13.59 (2×$CH_2CH_2CH_3$), 17.95 and 18.15 (2× $CH_2CH_2CH_3$), 22.82 (C-4CH_3), 36.04 and 36.29 (2×$CH_3CH_2CH_2$–C=O), 108.38 (C-8), 111.45 (C-10), 113.53 (C-6), 116.04 (C-3), 148.23 (C-4), 150.67 (C-9), 152.23 (C-5), 155.06 (C-7), 159.46 (C-2), 170.89 and 171.29 (2 × C=O); CI-MS, m/z (rel. int.): 333 $[M+1]^+$ (100), 263(9), 247(6), 193(2), 177(3), 105(19), 88(58), 86(6), 72(4) and 58(5).

5,6,7-Trimethoxy-4-methyl-2*H*-1-benzopyran-2-one (61). White solid, mp 116–117 °C, IR (KBr): 2942, 1720, 1599, 1552, 1444, 1405, 1380, 1359, 1294, 1201, 1154, 1107, 1076, 1017, 990, 916, 867, 819, 739 and 622 cm^{-1}; UV (MeOH): 320, 230 and 211 nm; ^1H NMR (300 MHz, $CDCl_3$): δ 2.87 (3H, s, CH_3), 3.87, 3.94 and 3.98 (9H, 3s, 3×-OCH_3), 6.05 (1H, s, H-3) and 6.65 (1H, s, H-8); ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.94 (CH_3), 56.12, 60.90 and 61.27 (3×OCH_3), 96.11 (C-8), 107.98 (C-10), 112.90 (C-3), 139.13 (C-4), 151.24 (C-9), 151.56, 153.52 and 156.19 (C-5, C-6 and C-7) and 160.80 (C-2); EI–MS, m/z (rel. int.): 250 $[M^+]$ (100), 235(73), 208(13), 207(80), 179(32), 177(8), 164(28) and 149(18).

2,4-Dihydroxy-5-(3-methyl-2-butenyl)phenylbenzyl ketone (70). White solid, mp 105–106 °C; IR (KBr): 3293, 3029, 2963, 2922, 1635, 1512, 1422, 1362, 1304, 1246, 1130, 1027, 850 and 695 cm^{-1}; UV (MeOH): 246, 313 and 320 nm; HR-MS, $C_{19}H_{20}O_3$ (M^+ 296.1418, calcd 296.1412); ^1H NMR (250 MHz, $CDCl_3$): δ 1.79 (6H, s, 2×CH_3), 3.26 (2H, d, J=6.95 Hz,-CH_2–CH=), 4.19 (2H, s,–$COCH_2$–), 5.23–5.31 (1H, m, =CH–CH_2–), 6.13 (1H, brs, C-4 OH), 6.34 (1H, s, H-3), 7.21–7.37 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′), 7.64 (1H, s, H-6) and 12.51 (1H, s, C-2 OH); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 17.85 and 25.83 (2×CH_3), 29.51 (–CH_2–CH=), 45.09 (–$COCH_2$–), 103.78 (C-3), 113.27 [$(CH_3)_2C$=CH–], 119.20 (C-5), 121.24 (C-6), 127.06 (C-4′), 128.76 (C-3′ and C-5′), 129.31 (C-2′ and C-6′), 131.93 [(= CH–CH_2–], 134.57 (C-1′), 135.38 (C-1), 161.59 (C-2), 164.08 (C-4) and 202.16 (C=O); EI–MS, m/z (% rel. int.): 296 $[M]^+$ (15.5), 279(3.1), 221(2.9), 206(14.6), 205(100.0), 203(6.8), 163(2.6), 161(2.2), 150(2.8), 149(27.1), 121(2.6), 105(1.1), 91(6.1) and 77(1.6); FAB-MS, m/z (% rel. int.): 297 $[M+H]^+$ (100).

2,4-Dihydroxy-3-(3-methyl-2-butenyl)phenyl benzyl ketone (71). White solid, mp 103–104 °C; IR (KBr): 3286, 2922, 1620, 1496, 1437, 1362, 1246, 1167, 1100, 1044, 848, 772 and 700 cm^{-1}; UV (MeOH): 215, 285, 318 and 326 nm; HR-MS, $C_{19}H_{20}O_3$ (M^+ 296.1418, calcd 296.1412); ^1H NMR (250 MHz, $CDCl_3$): δ 1.81 (6H, s, 2×CH_3), 3.42 (2H, d, J=7.18 Hz, –CH_2–CH=), 4.20 (2H, s, –$COCH_2$–), 5.24–5.27 (1H, m, =CH–CH_2–), 6.28 (1H, s, C-4 OH), 6.36 (1H, d, J=8.85 Hz, H-5), 7.22–7.35 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′), 7.64 (1H, d, J=8.88 Hz, H-6) and 13.0 (1H, s, C-2 OH); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 17.88 and 25.77 (2×CH_3), 29.88 (–CH_2–CH=), 44.78 (–$COCH_2$–), 107.91 (C-5), 117.25 [$(CH_3)_2C$=CH_2–], 121.71 (C-3), 127.0 (C-4′), 128.22 (C-3′ and C-5′), 128.70 (C-2′ and C-6′), 130.07 (C-6), 130.26 (–CH_2–CH=), 134.58 (C-1′), 135.55 (C-1), 161.63 (C-2), 163.15 (C-4) and 202.18 (C=O); EI–MS, m/z (% rel. int.): 296 $[M]^+$ (38.4), 279(2.5), 206(14.2), 205(97.7), 161(3.5), 150(10.0), 149(100.0), 105(2.0), 91(5.1) and 65(3.5).

2,4-Dihydroxy-3,5-di(3-methyl-2-butenyl)phenyl benzyl ketone (72). Green needles, mp 102–103 °C; IR (KBr): 3314, 2923, 1628, 1579, 1460, 1356, 1277, 1217, 1164, 1131, 1055, 962 and 727 cm^{-1}; UV (MeOH): 227, 289 and 336 nm; HR-MS, $C_{24}H_{28}O_3$ (M^+ 364.2029, calcd 364.2038); ^1H NMR (250 MHz, $CDCl_3$): δ 1.73 (6H, 2s, 2×CH_3), 1.80 (6H, brs, 2×CH_3), 3.24 and 3.41 (2d, 2H each, J=7.25 and 7.13 Hz, 2×–CH_2–CH=), 4.19 (2H, s, –$COCH_2$–), 5.19–5.31 (2H, m, 2×=CH–CH_2–), 6.21 (1H, s, C-4 OH), 7.24–7.36 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′), 7.47 (1H, s, H-6) and 13.00 (1H, s, C-2 OH); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 17.80, 17.88 and 21.82 (4×CH_3), 25.80 and 28.53 (2×–CH_2–CH=), 45.06 (–$COCH_2$–), 112.59 and 114.10 [2×$(CH_3)_2C$=CH–], 118.99 (C-6), 121.24 (2×=CH–CH_2–), 121.52 (C-3 and C-5), 126.93 (C-4′), 128.68 (C-3′ and C-5′), 129.22 (C-2′

and C-6′), 134.84 (C-1′), 135.34 (C-1), 160.09 (C-2), 161.57 (C-4) and 202.12 (C=O); EI–MS, *m/z* (% rel. int.): 364 $[M]^+$ (43.9), 347(2.7), 309(3.1), 274(20.7), 273(100.0), 271(4.9), 218(8.5), 217(55.6), 205(4.8), 162(4.7), 161(44.6), 149(8.0) and 91(6.2)

3,4-Dihydro-7-hydroxy-2,2-dimethyl-6-phenylacetyl-2*H*-1-benzopyran (73). White solid, mp 110–112 °C; IR (Nujol): 2924, 1639, 1463, 1377, 1347, 1255, 1170, 1150, 1117, 984 and 736 cm^{-1}; UV (MeOH): 236, 285 and 327 nm; ^1H NMR (250 MHz, $CDCl_3$): δ 1.34 (6H, s, 2×CH_3), 1.81 (2H, t, J=6.7 Hz, H-3), 2.72 (2H, t, J=6.7 Hz, H-4), 4.19 (2H, s, –$COCH_2$–), 6.31 (1H, s, H-8), 7.25–7.33 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′), 7.55 (1H, s, H-5) and 12.30 (1H, s, C-7 OH); ^{13}C NMR (62.8 MHz, $CDCl_3$): δ 21.75 and 26.95 (2×CH_3), 32.68 (C-3 and C-4), 44.64 (CO*C*H_2), 77.51 (C-2), 104.81 (C-8), 112.82 and 113.23 (C-10 and C-1′), 126.97 (C-4′), 128.67 (C-2′ and C-6′), 129.35 (C-3′ and C-5′), 131.90 (C-5) and 134.60 (C-6), 161.48 (C-9), 163.38 (C-7) and 201.62 (C=O); EI–MS *m/z* (% rel. int.): 296 $[M]^+$ (8), 204(100), 149(22), 121(4) and 44(12).

(±)-3,4-Dihydro-3,7-dihydroxy-2,2-dimethyl-6-phenylacetyl-2*H*-1-benzopyran (74). White solid, mp 97–98 °C; IR (KBr): 3524, 2928, 1643, 1491, 1356, 1302, 1235, 1128, 1064, 950 and 728 cm^{-1}; UV (MeOH): 236, 284 and 395 nm; HR-MS, $C_{19}H_{20}O_4$ (M^+ 312.1349, calcd 312.1362); ^1H NMR (300 MHz, $CDCl_3$): δ 1.35 and 1.37 (6H, 2s, 2×CH_3), 2.74 (1H, dd, J=4.80 and 15.97 Hz, H-4a), 3.03 (1H, dd, J=4.80 and 16.65 Hz, H-4b), 3.84 (2H, brs, C-3 OH and C-3H), 4.20 (2H, s, –$COCH_2$–), 6.03 (1H, s, H-8), 7.27–7.32 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′), 7.57 (1H, s, H-5) and 12.30 (1H, s, C-7 OH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 22.02 and 24.15 (2×CH_3), 30.49 (C-4), 44.71 (–CO*C*H_2–), 69.28 (C-3), 78.31 (C-2), 98.18 (C-8), 110.72 (C-10), 126.36 (C-1′), 126.99 (C-4′), 128.68 (C-3′ and C-5′), 129.32 (C-2′ and C-6′), 132.66 (C-5), 134.50 (C-6), 163.48 (C-7), 166.48 (C-9) and 201.73 (C=O); EI–MS, *m/z* (% rel. int.): 312 $[M]^+$ (15.3), 297(1.3), 279(1.1), 253(2.8), 222(13.8), 221(100.0), 204(2.4), 203(17.4), 163(3.9), 161(3.3), 150(1.6), 149(10.4), 122(1.9), 105(1.5), 91(4.9) and 77(1.1).

(±)-3,4-Dihydro-2,2-dimethyl-3-hydroxy-7-methoxy-6-phenylacetyl-2*H*-1-benzopyran (75). White solid, mp 112–113 °C; IR (KBr): 3431, 2923, 2853, 1734, 1665, 1611, 1573, 1494, 1210, 1136, 1031 and 730 cm^{-1}; UV data (MeOH): 234, 274 and 325 nm; ^1H NMR (300 MHz, $CDCl_3$): δ 1.32 and 1.35 (2s, 6H each, 2×CH_3), 2.66 (1H, dd, J=5.80 and 16.56 Hz, H-4a), 2.96 (1H, dd, J=4.78 and 16.54 Hz, H-4b), 3.78 (2H, bs, C-3 OH and C-3H), 3.84 (3H, s, –OCH_3), 4.27 (2H, s, –$COCH_2$–), 6.38 (1H, s, H-8), 7.18–7.31 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′) and 7.57 (1H, s, H-5). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 24.0 and 25.90 (2×CH_3), 29.44 (C-4), 49.90 (–CO*C*H_2–), 55.55 (OCH_3), 69.42 (C-3), 78.01 (C-2), 111.11 (C-10), 120.65 (C-8), 126.33 (C-4′), 127.55 (C-1′), 128.23 (C-3′ and C-5′), 129.56 (C-2′ and C-6′), 133.47 (C-5), 135.74 (C-6), 161.0 (C-7), 164.84 (C-9) and 197.89 (C=O); EI–MS, *m/z* (% rel. int.): 326 $[M]^+$ (3.2), 267(1.2), 236(16), 235(100), 233(1.7), 218(3.1), 217(20.5), 177(2.2), 175(3.2), 163(14.0), 138(2.5), 119(1.3), 118(1.2), 105(1.3), 91(5.6) and 77(1.2).

Acknowledgements

We thank Rita Colman and Arlette Verspeelt for technical assistance with the invasion assays, Georges De Bruyne for immunohistochemistry, Chris Dragonetti and Jean Roels Van Kerckvoorde for preparing the illustrations and Dr. Oliver Seitz (Max-Planck-Institute, Dortmund, Germany) for his assistance in getting the HRMS of our compounds recorded. We also thank the Danish International Development Agency (DANIDA, Denmark), Council of Scientific and Industrial Research (CSIR, New Delhi, India), Fonds voor Wetenschappelijk Onderzoek and Sportvereniging tegen Kanker (Brussels, Belgium) for financial assistance.

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