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Bioactive 4-Substituted-6-methyl-2-pyrones with Promising Cytotoxicity against A2780 and K562 Cell Lines

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Abstract—Bioactive synthetic 4-substituted-6-methyl-2-pyrones are reported. Various 4-substitutents have been incorporated using Pd-catalysed carbon–carbon bond coupling procedures. Preliminary screening of the 2-pyrones against human ovarian carcinoma (A2780) and human chronic myelogenous leukaemia (K562) cell lines show that 4-alkynyl-6-methyl-2-pyrones have excellent potential as anticancer agents. The pyrones demonstrate broad spectrum antimicrobial activities.

The 2-pyrone sub-unit[1] is an important synthetic building block[2] found in numerous biologically active natural products,[3] for which there has been considerable recent interest.[4] Of interest to us are several reports on cytotoxic pyrones from natural sources.[5] The Bufadienolides (**1**) provide an excellent example with a diverse range of biological activities (Fig. 1).[6]

One particular drawback of such natural products is their molecular complexity and low yields from natural extraction/isolation procedures. We therefore had a desire to synthesise much simpler 2-pyrone systems with a view to identifying promising: (1) bioactivity against cancer cells; and (2) antimicrobial activity. A potential and promising field of study is in the application of antimicrobial pyrones for use in agriculture, as pesticides or herbicides. Fusapyrone **2**[7] is a recent example of a 2-pyrone with considerable antifungal activity, low phytotoxicity and mycotoxicity.

We herein report on the synthesis and biological evaluation of relatively simple 4-alkyl/aryl/alkenyl/alkynyl-6-methyl-2-pyrones **3**.

An efficient synthetic methodology that allows for the facile introduction of a plethora of alkyl, aryl, alkenyl and alkynyl substituents was required. For this we focused our attention on Pd-catalysed C–C bond forming processes, as we felt that the range of substituents that could be incorporated would be greater and accomplishable in higher yields than the more laborious traditional methods for introducing substituents into the pyrone ring system. There are surprisingly few reports on direct Pd-catalysed coupling of nucleophiles, such as boranes, boronic acids, benzodioxaboroles and alkynylcopper reagents (generated under Songashira conditions), onto the electrophilic pyrone ring system. There are two reports on Pd-catalysed coupling of alkyl

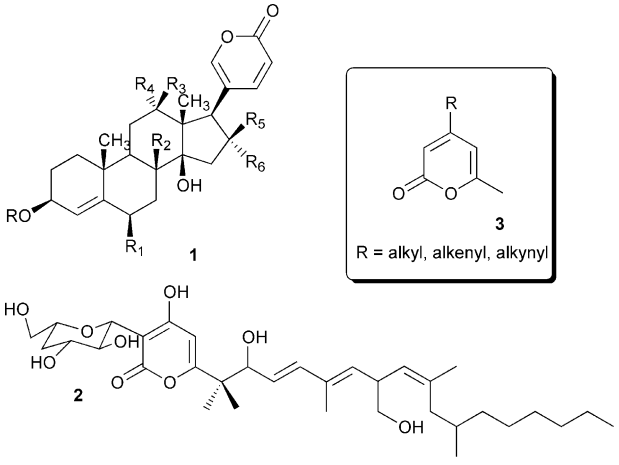

Figure 1.

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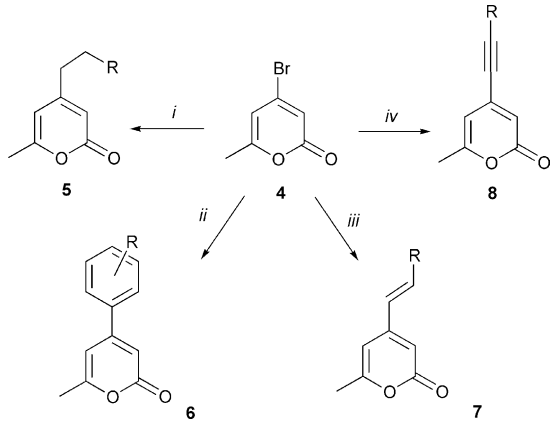
and alkynyl zinc reagents to 4-bromo-6-methyl-2-pyrone.[8] In particular, an efficient synthesis to the cockroach sex pheromone, Supellapyrone (5-(2′*R*,4′*R*-dimethylheptanyl)-3-methyl-2-pyrone) has been reported using an alkylzinc reagent as the key step, which allowed the *syn* stereochemistry of the alkyl chain to be deduced.[8a,b]

We chose 4-bromo-6-methyl-2-pyrone **4** (available in 79% yield using PBr_3 in DMF at 70 °C from commercially available 4-hydroxy-6-methyl-2-pyrone) as the starting material for the coupling reactions (Scheme 1).

Coupling with trialkylboranes

The synthesis of 4-alkyl-6-methyl-2-pyrones (**5**) were initially envisaged as being easily accessible from alkylboronic acids and alkylbenzooxaboroles. However, several Pd-catalysts and conditions (solvents, base and reaction temperature) were unsuccessful. To our delight, we were able to couple trialkylboranes with **4** using [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) [Pd(dppf)Cl_2] as the catalyst, thallium carbonate as a mandatory base in a THF–H_2O solvent mixture, which allowed the reactions to be run at 25 °C (Table 1). The yields of cross-coupled products (**5a–h**) were satisfactory and thus at this point we did not investigate more efficient catalysts or reaction conditions.



Scheme 1. Pd-catalysed C–C bond couplings of **4**: (i) BH_3·THF, alkene, 0 °C *then* Pd(dppf)Cl_2, Tl_2CO_3, THF/H_2O, 25 °C; (ii) Pd($OAc)_2$, PPh_3, arylboronic acid, Na_2CO_3, benzene, Δ; (iii) Pd($OAc)_2$, PPh_3 RCH=CHM (*E*), Na_2CO_3, benzene, Δ; (iv) Pd/C, PPh_3, CuI, Et_3N, CH_3CN, Δ.

Coupling with arylboronic acids

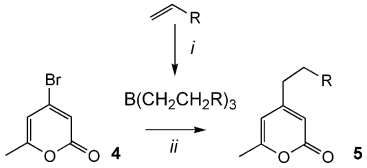
The Suzuki coupling of **4** with several boronic acids proceeded well using Pd(0) based catalysts, such as tetrakis-(triphenylphosphine)palladium(0) [$(PPh_3)_4$Pd] and a palladium(II)acetate [Pd($OAc)_2$]/triphenylphosphine combination. The latter catalyst proved more versatile and less sensitive to air. The bases generally used in Suzuki couplings are NaOEt and NaOH, but these were far to strong for the 2-pyrone sub-unit to withstand, due to basic hydrolysis.

We found that use of Na_2CO_3 was of paramount importance to the yields of **6**. In Table 2, coupling of several boronic acids can be found. The electron-donating arylboronic acids (entries 2 and 4, Table 2) provided the coupled products in generally good yields. Changing the ligand to trifurylphosphine improved the yields. Although little problems were seen with the chloro-substituent (entry 3, Table 2), changing to the more electron withdrawing nitro and formyl substituents had a dramatic effect on the yields (entries 5 and 6, Table 2). For the latter, the P(furyl)$_3$ ligand improved the yield to 43% from 20%.

Coupling with alkenylboronic acids

The initial investigations into coupling alkenylbenzodioxaboroles with **4** under Pd(0) catalysis gave the coupled products in 30–40% yields. However, we felt these reactions were cumbersome and the air-sensitive benzodioxaboroles made them unattractive. On the other hand, coupling of **4** to alkenylboronic acids were much more facile and yields of the 4-alkenyl-2-pyrones improved dramatically (Table 3). As for the arylboronic acids, use of Na_2CO_3 proved a mandatory base. It is of interest that we have so far been unable to synthesise

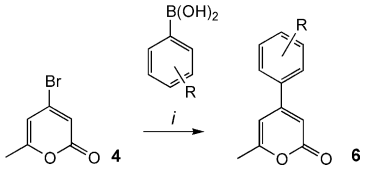
Table 1. Coupling of various alkenes with **4**[a]



Entry	Pyrone (R)	Yield
1	H, **5a**	56
2	CH_3CH_2–, **5b**	60
3	$CH_3(CH_2)_2$–, **5c**	50
4	$CH_3(CH_2)_3$–, **5d**	46
5	$CH_3(CH_2)_4$–, **5e**	54
6	$CH_3(CH_2)_5$–, **5f**	41
7	Ph–, **5g**	59
8	CH_3CH_2O–, **5h**	32

[a]Conditions: (i) BH_3·THF, alkene, 0 °C, 1 h; (ii) Pd(dppf)Cl_2 (5 mol%), Tl_2CO_3, THF/H_2O, 25 °C.

Table 2. Coupling of various arylboronic acids with **4**[a,b]



Entry	Pyrone (R)	Yield
1	H–, **6a**	56 (81)
2	*p*-CH_3–, **6b**	52 (76)
3	*p*-Cl–, **6c**	53
4	*p*-CH_3O–, **6d**	62 (86)
5	*m*-NO_2–, **6e**	0
6	*p*-CHO–, **6f**	20 (43)

[a]*Conditions:* (i) Pd($OAc)_2$ (6 mol%), PPh_3 (18 mol%), arylboronic acid, Na_2CO_3, benzene, Δ, 6 h.
[b]Using P(furyl)$_3$ (18 mol%) as the ligand in place of PPh_3, yields in parentheses.

these analogues using Heck coupling of **4** with alkenes under a variety of standard conditions.

Coupling with alkynes (Sonogashira)

We have recently found that terminal acetylenes couple to **4** using Sonogashira coupling. Exploration of a range of catalyst systems led to the surprising finding that Pd/C efficiently catalyses the coupling of **4** with terminal acetylenes. In Table 4 can be found the yields of the 4-alkynyl-6-methyl-2-pyrones from several terminal acetylenes.

The TMS derivative **8a** was synthesised with a view to accessing the free alkyne **8k**. Silyl cleavage is best performed using tetrabutylammonium fluoride (TBAF) in THF (1 M) at −78 °C. After 2 h **8k** is isolated in 80% yield.[9]

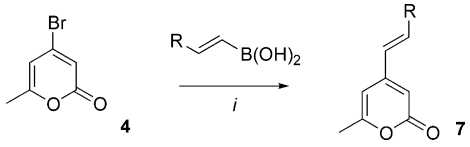
Biological Results

The growth inhibitory activities of the 2-pyrones were determined in the A2780 human ovarian carcinoma and K562 human chronic myelogenous leukaemia cell lines using an in vitro cell culture system (MTT assay).[10] The assay itself is based on the reduction of 3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyl-tetrazolium bromide (MTT, yellow colour) by mitochondrial dehydrogenases of metabolically active cells to a purple-blue formazan. The results are shown in Table 5. Comparable potent inhibitors against these cell lines have been reported.[11]

From the data in Table 5, it is evident that the antiproliferative activity is variously manifested in the two cell lines and it is possible to discern some quite prominent structure–activity relationships. The compounds that were not active at IC_{50} < 50 μM were those which contained the 4-alkyl and 4-alkenyl substitutents (**5a–d**, **5g–h**, **7c–e**), although some activity was observed for the heptyl derivative **5e** which had an IC_{50} (K562) of 28.7 μM and IC_{50} (A2780) of 41.8 μM. The most potent compounds in this series were the 4-alkynyl derivatives (**8b–g** and **8k**). The most potent derivative against A2780 was phenylethynyl derivative **8b** with an IC_{50} of 1.8 μM. Compound **8b** was also active against K562 (IC_{50} = 4.0 μM). Increasing the alkynyl chain from pentynyl through to a heptynyl chain (**8c–e**) sees an improvement in activity against the K562 cell line, peaking at **8e**.

By extending the alkynyl chain by one carbon to **8f**, we see a fall off in activity. A slightly different trend for the A2780 cell line is observed, although the activity again peaks with **8e**. A somewhat striking result is observed by comparing the activities of **8f** for both cell lines [IC_{50} = (A2780) 3.4 μM and (K562) 20.3 μM]. Such differences are observed with alkynyl derivatives **8c–f** for both cell lines to a more or lesser degree. Two further potent analogues, in addition to **8b**, are **8g** and **8k** with an IC_{50} (A2780) of 2.1 and 3.1 μM and IC_{50} (K562) of 4.0 and 2.0 μM, respectively.

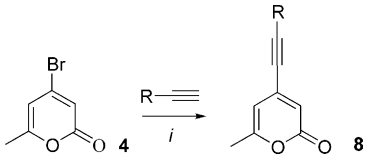
Table 3. Suzuki coupling of alkenylboronic acids with **4**[a]



Entry	Pyrone (R)	Yield
1	$CH_3(CH_2)_2$–, **7a**	58
2	$CH_3(CH_2)_3$–, **7b**	66
3	$CH_3(CH_2)_4$–, **7c**	60
4	$CH_3(CH_2)_5$–, **7d**	56
5	Ph–, **7e**	27

[a]*Conditions:* (i) $Pd(OAc)_2$ (6 mol%), PPh_3 (18 mol%), alkenylboronic acid, Na_2CO_3, benzene, Δ, 6 h.

Table 4. Sonogashira coupling of terminal alkynes with **4**[a]



Entry	Pyrone (R)	Yield
1	$(CH_3)_3Si$–, **8a**	82
2	Ph–, **8b**	74
3	$CH_3(CH_2)_2$–, **8c**	72
4	$CH_3(CH_2)_3$–, **8d**	77
5	$CH_3(CH_2)_4$–, **8e**	79
6	$CH_3(CH_2)_5$–, **8f**	73
8	$THPOCH_2$–, **8g**	81
9	*p*-NH_2-Ph–, **8h**	21
10	*p*-NHAc-Ph–, **8i**	95
11	*p*-NO_2-Ph–, **8j**	95
See text	H–, **8k**	*See text*

[a]*Conditions:* (i), 10% Pd/C (20 wt%), PPh_3 (25 wt%), dry Et_3N, dry CH_3CN (2.5:1.5), CuI (4 mol%) under N_2, 3 h.

Table 5. In vitro IC_{50} cytotoxicity results against A2780 and K562 cell lines[10]

R	A2780 (μM)	K562 (μM)
Ethyl, **5a**	> 50	> 50
Butyl, **5b**	> 50	> 50
Pentyl, **5c**	> 50	> 50
Hexyl, **5d**	> 50	> 50
Heptyl, **5e**	41.8	28.7
2-Phenylethyl, **5g**	> 50	> 50
Ethoxyethyl, **5h**	> 50	> 50
Ph, **6a**	> 50	> 50
p-CH_3–, **6b**	> 50	> 50
p-Cl–, **6c**	> 50	> 50
p-CH_3O–, **6d**	> 50	> 50
p-CHO–, **6f**	19.2	26.0
Hexenyl, **7c**	> 50	> 50
Heptenyl, **7d**	> 50	> 50
Phenylethenyl, **7e**	> 50	> 50
Phenylethynyl, **8b**	1.8	4.0
Pentynyl, **8c**	4.8	17.8
Hexynyl, **8d**	7.0	15.5
Heptynyl, **8e**	2.9	11.1
Octynyl, **8f**	3.4	20.3
$THPOCH_2$, **8g**	2.1	4.0
Ethynyl, **8k**	3.1	2.0

Table 6. Antimicrobial activities of 4-substituted-6-methyl-2-pyrones[a]

Compd	*Bacillus subtilis*[b]	*Escherichia coli*[b]	*Staphylococcus aureus*[b]	*Candida albicans*[c]	*Saccharomyces cerevisiae*[c]	*Schizosaccharomyces pombe*[c]
5a	0	21 (23.8)	0	0	0	0
5b	20 (22.7)	22 (25.0)	0	0	0	0
5c	23 (26.1)	22 (25.0)	0	0	0	22 (25.0)
5d	21 (23.9)	25 (28.4)	0	11 (12.5)	0	26 (29.5)
5e	16 (18.2)	17 (19.3)	14 (15.9)	16 (18.2)	5 (5.7)	30 (34.1)
5f	19 (21.6)	22 (25.0)	18 (20.5)	20 (22.7)	0	30 (34.1)
5g	18 (20.5)	20 (22.7)	0	0	0	10 (11.4)
5h	NT	NT	NT	NT	NT	0
6a	18 (20.5)	20 (22.7)	18 (20.5)	0	0	0
6b	19 (21.6)	18 (20.4)	0	0	0	0
6c	18 (20.5)	20 (22.7)	20 (22.7)	0	0	0
6d	17 (19.3)	20 (22.7)	0	0	0	20 (22.7)
6f	18 (20.5)	15 (17.0)	18 (20.5)	0	0	14 (15.9)
7a	16 (18.2)	19 (21.6)	20 (22.7)	16 (18.2)	0	28 (31.8)
7b	18 (20.5)	18 (20.5)	0	24 (27.3)	0	40 (45.5)
7c	18 (20.5)	18 (20.5)	17 (19.3)	21 (23.9)	0	34 (38.6)
7d	18 (20.5)	20 (22.7)	18 (20.5)	20 (22.7)	0	29 (32.9)
7e	18 (20.5)	17 (19.3)	0	0	0	18 (20.5)
8b	19 (21.6)	20 (22.7)	0	0	0	37 (42.0)
8c	18 (20.5)	22 (25.0)	21 (23.9)	11 (12.5)	0	29 (32.9)
8d	17 (19.3)	20 (22.7)	0	20 (22.7)	0	45 (51.1)
8e	19 (21.6)	19 (21.6)	21 (23.9)	22 (25.0)	0	50 (56.8)
8f	17 (19.3)	17 (19.3)	18 (20.5)	0	0	48 (54.5)
8g	20 (22.7)	17 (19.3)	30 (34.1)	13 (14.8)	0	18 (20.5)
8i	6 (6.8)	0	16 (18.2)	0	0	0
8j	6 (6.8)	0	0	0	0	9 (10.2)
8k	17 (19.3)	16 (18.2)	30 (34.1)	36 (40.9)	40 (45.4)	40 (45.5)

[a]Each cellulose disk contained 200 μg of the test compound. NT, not tested. Radii/mm (percent) of inhibition.
[b]After 96 h incubation.
[c]After 48 h incubation.

The aryl derivatives (**6a**–**d**) showed no inhibitory activity at a concentration of <50 μM. However, formyl substituted derivative **6f** demonstrated promising activity against both cell lines (IC_{50} = (A2780) 19.2 μM and (K562) 26.0 μM). This result is significant in that it illustrates the effect of making a simple change in substituent can have such a dramatic outcome.

The antimicrobial results can be seen in Table 6. Potent control compounds were used for comparison in these assays.[12] We have detailed a comprehensive set of data for the 2-pyrones against three bacteria and three yeasts. The assay used was a standard agar diffusion method as previously described.[13]

With the exception of compounds **8i** and **8j** all the pyrones tested are active against *B. subtilis* and *E. coli*. The most potent pyrones were the pentyl and hexyl derivatives **5c** and **5d**, although both of these derivatives demonstrate no activity towards *S. aureus*. The most potent pyrones against *S. aureus* by some distance were **8g** and **8k**. Indeed **8k** was also potent towards the yeasts, *C. albicans*, *S. cerevisiae* and *S. pombe*, whereas **8g** showed only modest activity against these yeasts. With the exception of **8k**, insignificant activity was observed for the pyrones against *S. cerevisiae*— an intriguing result! In stark contrast, potent activity was observed against *S. pombe* for many of these pyrones. The most potent analogue was heptynyl derivative **8e**. The results clearly suggest that the presence of a hydrophobic 4-substituent is key to inhibitory activity.

In conclusion we have synthesised a plethora of 4-alkyl/aryl/alkenyl/alkynyl-6-methyl-2-pyrones using Pd-catalysed coupling procedures. These studies represent the first comprehensive investigations into such coupling processes for 4-bromo-2-pyrones and important reaction conditions are given. The 4-alkynyl-6-methyl-2-pyrones have been identified as promising new cytotoxic agents against both A2780 and K562 cell lines and details of significant antimicrobial activities have been presented. In short the 4-substituted pyrones are extremely bioactive in a range of species and in due course we aim to uncover their mode(s) of action.

Acknowledgements

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10. (a) The standard assay for cytotoxicity: The assay used a standard 96 well format (Bibby Sterilin). Between 400 and 1000 cells were plated into each well, in a total volume of 50 μL. The cells were allowed to recover for 24 h before 150 μL of medium containing the drug was added to each well. The control wells just received 150 μL of medium alone. Each drug dose was represented by three wells. The plates were then incubated at 37 °C in a 5% CO_2 atmosphere for 5 days. After incubation, 50 μL of MTT solution (3 mg/mL) was added to each well and the plate returned to the incubator for 3 h. After this time, the medium and excess MTT was aspirated from each well and the formazan crystals were solubilised in 200 μL of DMSO. The plate was then read using a multiscan microplate reader (Titretech, Labsystems UK Ltd) at 540 nm with subtraction at 620 nm to allow for turbidity. The resultant output was processed and the percentage growth inhibition for each dose calculated, each experiment was performed in duplicate. Mean and standard deviations for each dose were calculated from duplicate experiments. Based on a standard assay: Mossmann, T. *J. Immunol. Methods* **1983**, *63*, 55. (b) Edmondson, J. M.; Armstrong, L. S.; Martinez, A. O. *J. Tissue Culture Methods* **1988**, *11*, 15.
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12. (a) An antibiotic 'ring', which contained several known potent anti-bacterial agents (Novobiocin, Penicillin G, Streptomycin, Tetracyline, Chloroamphenicol, Erythromycin, Fusidic acid and Methicillin), was used as the control in the bacterial assays (individual discs contain 1 unit). Zones of inhibition (~5–8 mm) were observed for these compounds. (b) Squalestatin S1 (Zaragozic acid A), an extremely potent yeast inhibitor, was used as a control in the yeast assay (200 μg/disk). The zones of inhibition observed against *C. albicans*, *S. cerevisiae* and *S. pombe* were 27, 27 and 37 mm, respectively.
13. The antimicrobial assays were performed as previously described: Fairlamb, I. J. S.; Dickinson, J. M.; Higson, S.; Grieveson, L.; O'Connor, R.; Marin, V. *Bioorg. Med. Chem.* **2002**, *10*, 2641 Each disk contained 20 μL of a 10 mg/mL solution of the test compound in DMSO, ethanol or H_2O to give a final concentration of 200 μg/disk.