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Efficient synthesis of substituted 7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones and evaluation of their in vitro antiproliferative/cytotoxic activities

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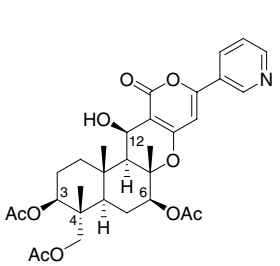
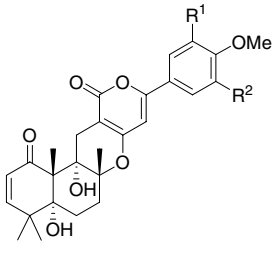
Abstract—Substituted 7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones and related heterocycles **3** were synthesized through an efficient domino Knoevenagel condensation/6π-electron electrocyclization. In vitro antiproliferative/cytotoxic activity evaluation was performed with human SH-SY5Y neuroblastoma cells and revealed IC_{50} values ranging from 6.7 to >200 μM. The compound that was most cytotoxic to the neuroblastoma cells, that is, 2-isobutyl-3-isopropyl-7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-one (**3a**), also exhibited necrotic effects on the human IPC melanoma cells.

In several papers Omura et al. have reported on the isolation of a number of compounds with novel structural features from the culture broth of *Aspergillus fumigatus*.[1] The structure elucidation of the new natural products that were named pyripyropenes A–R revealed that they are characterized by a decahydro-2*H*,11*H*-naphtho[2,1-*b*]pyrano[3,4-*e*]pyran-11-one skeleton. The compounds with up to eight stereogenic centers differ only in respect to the presence or absence of functional groups at C-3, C-4, C-6, and C-12. The investigation of their biological activity identified pyripyropenes to be the most potent naturally occurring, biologically available inhibitors of acyl-CoA:cholesterol acyltransferase (ACAT).[1] The inhibition of ACAT, the enzyme responsible for intracellular esterification of cholesterol, represents a promising new approach to the prevention of atherosclerosis.[2] Related natural products like arisugacins and territrems have been reported to possess important biological activities, for example, inhibition of acetylcholinesterase.[3]

In this context the investigation of the biological activity of simple model compounds is of particular interest. While it was found that tricyclic compounds with a tetrahydro-1*H*,7*H*-pyrano[4,3-*b*] [1] benzopyran-1-one skeleton exhibit both in vitro and in vivo cytotoxic activity,[4] the comparative investigation of the biological activity of bicyclic 2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones with varying substituents at C-2, C-3, and C-7 remains to be done yet. For this purpose we have synthesized a small library of novel mono-, di-, and trisubstituted 7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones **3** by an efficient domino Knoevenagel condensation/6π-electron electrocyclization reaction. The compounds were then evaluated for their in vitro antiproliferative/cytotoxic activity using human SH-SY5Y neuroblastoma and IPC melanoma cells.



Pyripyropene A



R^1 = H, R^2 = OMe Arisugacin A
R^1 = H, R^2 = H Arisugacin B
R^1 = R^2 = OMe Territrem B

Keywords: Heterocycles; Domino reaction; Cytotoxicity; Apoptosis; Substituent effects.
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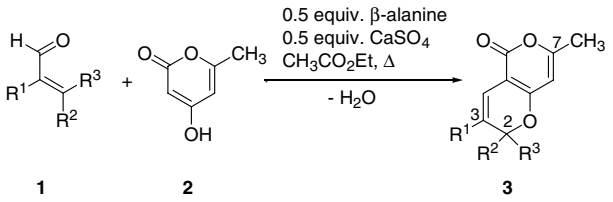
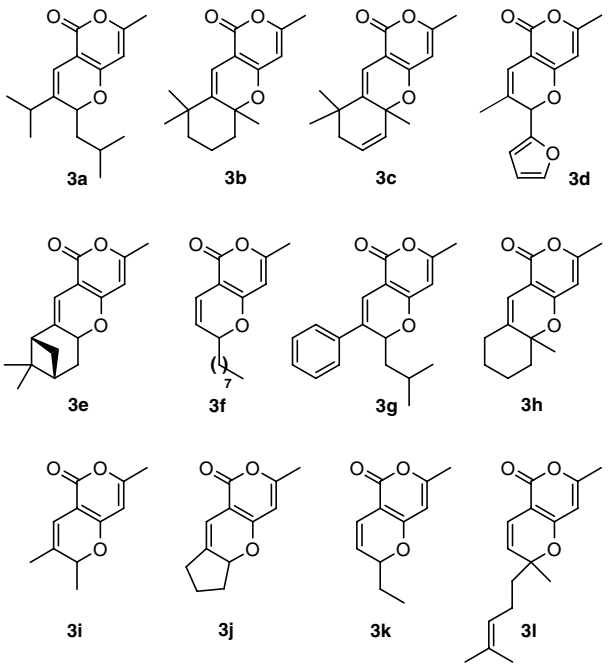
7-Methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones and related heterocycles **3** can be synthesized in a domino process[5] by reaction of α,β-unsaturated aldehyde **1** with 4-hydroxy-6-methyl-2*H*-pyran-2-one (**2**).[6]



1 **2** **3**

In the presence of an amino acid such as L-proline or an amine such as piperidine a Knoevenagel condensation[7] occurs, first yielding a 1-oxatriene. Usually, the 1-oxatriene formed cannot be isolated, but subsequently reacts in a 6π-electron electrocyclic ring closure reaction[8] giving the heterocycle **3**. In a number of cases the yields of the heterocycles **3** could be improved by performing a two-step transformation. Following this protocol the pyrone **2** is reacted with a preformed α,β-unsaturated iminium salt available by reaction of the α,β-unsaturated aldehyde **1** with piperidine/Ac_2O.[9] The heterocycles can also be obtained in a single synthetic operation if the reaction of the pyrone **2** with the α,β-unsaturated aldehyde **1** is performed in a sealed tube in the presence of piperidinium acetate.[9a]

We found that the heterocycles **3** can be most efficiently synthesized in one step by direct reaction of 1.0 equiv aldehyde **1** with 1.1 equiv pyrone **2** in the presence of an amine or an amino acid and a dehydrating agent. The best results were obtained using β-alanine and calcium sulfate in boiling ethyl acetate.[10] Under these reaction conditions 2-isopropyl-5-methyl-2-hexenal (**1a**), β-cyclocitral (**1b**), safranal (**1c**), 2-methyl-3-(2-furyl)-propenal (**1d**), (-)-myrtenal (**1e**), (*E*)-2-undecenal (**1f**), 5-methyl-2-phenyl-2-hexenal (**1g**), 2-methyl-1-cyclohexene-1-carboxaldehyde (**1h**), (*E*)-2-methyl-2-butenal (**1i**), 1-cyclopentene-1-carboxaldehyde (**1j**), (*E*)-2-pentenal (**1k**), and citral (**1l**) were reacted with 4-hydroxy-6-methyl-2*H*-pyran-2-one (**2**) to give a small library of 7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones and related heterocycles **3**.[10,11] These compounds could be obtained at reaction times ranging from 1 to 25 h and yields from 28% to 96% depending on the structure of the α,β-unsaturated aldehyde **1**. Reactions of sterically hindered aldehydes like β-cyclocitral (**1b**) (Table 1, Entry 2) and safranal (**1c**) (Table 1, Entry 3) resulted in low yields and long reaction times. All the other heterocycles **3** could be obtained in good to very good yields (57–96%). The reactions can be accelerated by working under microwave conditions.[12] Thus we were able to synthesize not only the tricyclic compounds **3b**,**c**,**e**,**h**,**j** but also the bicyclic ones (**3a**,**d**,**f**,**g**,**i**,**k**,**l**) with different substituents at C-2 and C-3 for the evaluation of their in vitro antiproliferative/cytotoxic activities.



Cells were cultured and the bioassay conducted as described by Rösner et al. with just minor modifications.[13,14] Each compound was tested in four replicates per dose and its relative antiproliferative/cytotoxic activity expressed as the mean percent inhibition of proliferation according to the following equation:

$$x = \frac{(\text{mean control prolif. ind.}) - (\text{mean treated prolif. ind.})}{(\text{mean control prolif. ind.})} \times 100\%.$$

Data are presented in Table 2. IC_{50} values (concentrations required for inhibiting cellular proliferation by 50%) were determined graphically.

Table 1. Synthesis of heterocycles **3** by domino Knoevenagel condensation/6π-electron electrocyclization of α,β-unsaturated aldehydes **1** with 4-hydroxy-6-methyl-2*H*-pyran-2-one (**2**)

Entry	Compound **1**	Reaction time (h)	Compound **3**[a]	Yield (%)[b]
1	**a**	25	**a**	84
2	**b**	25	**b**	28
3	**c**	20	**c**	28
4	**d**	22	**d**	57
5	**e**	15	**e**	93
6	**f**	4	**f**	96
7	**g**	6	**g**	84
8	**h**	7	**h**	63
9	**i**	1	**i**	73
10	**j**	8	**j**	63
11	**k**	2	**k**	76
12	**l**	2	**l**	94

[a] All new products (**3a**–**l**) were fully characterized by ^1H NMR, ^{13}C NMR, gCOSY, DPFGNOE, gHSQC, gHMBC, MS, IR, UV spectroscopy, and combustion analysis.

[b] Yields refer to analytically pure compounds.

Table 2. Antiproliferative/cytotoxic activities of compounds **3a–l** on the human SH-SY5Y neuroblastoma cell line in vitro

Compound **3**	Mean % inhibition of proliferation ± SE[a]						Cytotoxicity IC_{50} (μM)
	50 μg/mL	25 μg/mL	12.5 μg/mL	6.25 μg/mL	3.13 μg/mL	1.56 μg/mL	
a	95.71 (±8.7)	96.64 (±7.9)	94.00 (±1.9)	93.79 (±1.9)	87.46 (±3.2)	38.48 (±6.6)	6.7
b	75.12 (±13.8)	74.16 (±12.6)	72.91 (±12.7)	18.42 (±11.1)	1.11 (±7.7)	8.34 (±5.9)	31
c	84.79 (±10.0)	79.85 (±9.5)	51.91 (±14.1)	16.95 (±4.3)	6.01 (±4.4)	n.d.	47
d	64.44 (±12.0)	64.26 (±11.3)	50.65 (±15.5)	30.91 (±9.7)	21.61 (±8.2)	n.d.	49
e	84.13 (±11.4)	67.78 (±13.5)	24.38 (±15.9)	n.d.	n.d.	n.d.	70
f	83.28 (±11.0)	60.12 (±12.9)	21.07 (±5.6)	n.d.	n.d.	n.d.	76
g	81.68 (±12.1)	45.74 (±13.5)	14.59 (±7.9)	n.d.	n.d.	n.d.	93
h	60.58 (±10.0)	11.40 (±6.7)	1.18 (±10.8)	n.d.	n.d.	n.d.	185
i	53.82 (±13.5)	43.79 (±14.0)	n.d.	n.d.	n.d.	n.d.	200
j	51.19 (±9.8)	14.92 (±12.7)	19.27 (±12.1)	n.d.	n.d.	n.d.	252
k	43.70 (±15.8)	4.95 (±6.0)	3.62 (±5.2)	n.d.	n.d.	n.d.	>200
l	37.41 (±11.8)	30.39 (±9.7)	n.d.	n.d.	n.d.	n.d.	>200

[a] Values are given as means of four experiments, standard error is provided in parentheses (n.d., not determined).

The present work studied the effects of modifying the functionalities associated with positions C-2 and C-3 in the 7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-one skeleton on the proliferation of human SH-SY5Y neuroblastoma cells in vitro. From the antiproliferation/cytotoxicity data presented in Table 2 it is evident that a modification of the substituents at these two positions dramatically altered the cytotoxic potencies of the molecules as revealed by the wide range in IC_{50} values varying from 6.7 μM for compound **3a** to >200 μM for compounds **3j–l**.

Compounds **3a** and **3b** which inhibited proliferation of human SH-SY5Y neuroblastoma cells at concentrations lower than 12.5 μg/mL induced a rounded morphology in the cells with subsequent detachment from the extracellular matrix (Fig. 1C and D). The compound with the most potent cytotoxic effects on the neuroblastoma cells was 2-isobutyl-3-isopropyl-7-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-one (**3a**) with an IC_{50} value of 6.7 μM. This compound also produced rounding, loss of adhesion, and necrosis in human IPC melanoma cells at 12.5 μg/mL (Fig. 1 H).

Similar antiadhesion/cytotoxicity effects on the neuroblastoma cells in vitro have been reported for some natural products, for example, dibenzyltrisulphide and organophosphate insecticides.[15]

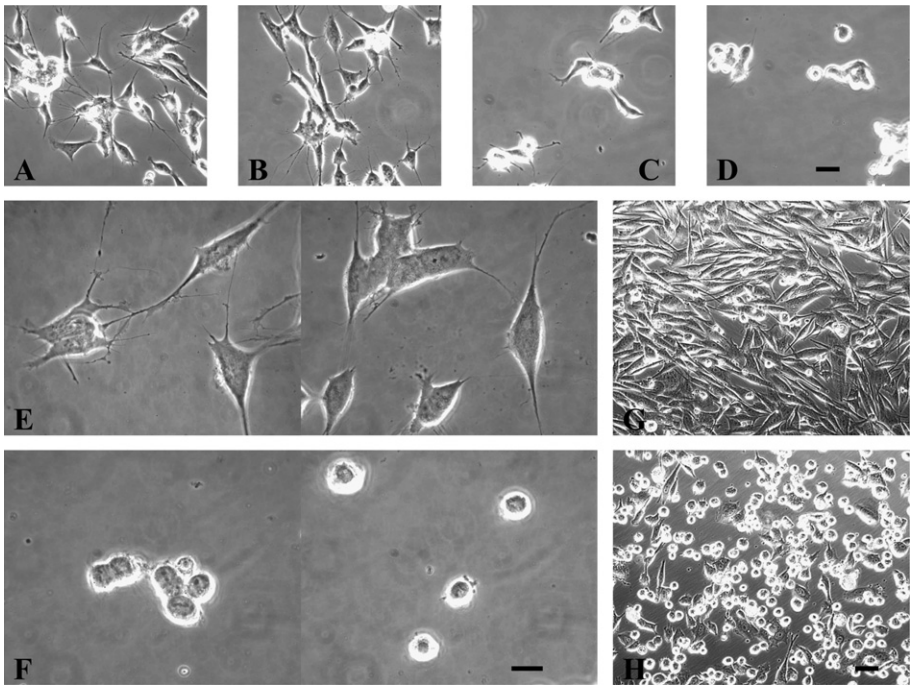


Figure 1. Different cytotoxicities of the related compounds **3a**, **3b**, and **3k**. Human SH-SY5Y neuroblastoma cells (A–F) and IPC melanoma cells (G and H) were treated with 12.5 μg/mL (fin. conc.) of the compounds for 3 h. (A and E) Untreated SH-SY5Y control cells. (B) Compound **3k**, no effect. (C) Compound **3b**, about 50% of the cells have lost adhesion and are rounded. (D and F) Compound **3a**, almost all cells have lost adhesion, appear shrunken and rounded, and contain large vesicles. (G) Untreated IPC control cells. (H) Compound **3a**, the majority of cells have lost adhesion and are rounded, many of them necrotic. The bars correspond to 30 μm (D), 20 μm (F), and 40 μm (H), respectively.

The above-mentioned cell biological effects appear to be similar to those associated with the phosphorylation of tyrosine amino acid residues in the integrin binding domain of some cancer cells.[16] The control cells and those treated with non-cytotoxic compounds remain attached to the extracellular matrix of the bottom while proliferating (Fig. 1A and E).

In summary, it can be concluded that cytotoxic activity is not only exhibited—as has already been known—by the tricyclic tetrahydro-1*H*,7*H*-pyrano[4,3-*b*][1]benzopyran-1-ones, but also by the bicyclic 2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones. Here, we can see that the nature of the substituents at C-2 and C-3 has a strong influence on the cytotoxicity of the 2*H*,5*H*-pyrano[4,3-*b*]pyran-5-ones. This is why we propose to undertake further synthetic modifications at positions C-2 and C-3 in the 2*H*,5*H*-pyrano[4,3-*b*]pyran-5-one skeleton in order to synthesize more potent cytotoxic molecules for anticancer evaluation.

Acknowledgment

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10. General procedure for the synthesis of heterocycles **3**: 1.39 g (11.0 mmol) 4-hydroxy-6-methyl-2*H*-pyran-2-one (**2**), 445 mg (5.0 mmol) β-alanine, 681 mg (5.0 mmol) anhydrous calcium sulfate, the α,β-unsaturated aldehyde **1** (10 mmol), and 50 mL dry ethyl acetate were heated under reflux until complete consumption of the aldehyde **1** (TLC control; petroleum ether/diethyl ether = 1:1). Reaction times are given in Table 1. After cooling to room temperature, the reaction mixture was washed with 25 mL of a saturated aqueous sodium hydrogen carbonate solution. The aqueous layer was extracted three times with 50 mL of ethyl acetate and the combined organic layers were washed with 25 mL brine, dried over magnesium sulfate, and concentrated in vacuo. The crude product was purified by flash chromatography on neutral aluminum oxide (deactivated with 19% water) (petroleum ether/diethyl ether = 1:1) or silica gel (petroleum ether/diethyl ether = 2:1) (compounds **3b** and **3c**) to give analytically pure heterocycles **3**.
11. Analytical and spectroscopic data for **3f**: white solid; mp = 42 °C; R_f = 0.50 (petroleum ether/diethyl ether = 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.87 (br t, 3H; 8′-H_3), 1.10–1.60 (m, 12H; 2′H_2 to 7′-H_2), 1.60–1.80 (m, 2H; 1′-H_2), 2.20 (s, 3H, 7-CH_3), 4.98 (m, 1H, 2-H), 5.40 (dd, $^3J_{3\text{-H},4\text{-H}}$ = 10.1 Hz, $^3J_{3\text{-H},2\text{-H}}$ = 3.2 Hz, 1H, 3-H), 5.77 (s, 1H, 8-H), 6.42 (d, $^3J_{4\text{-H},3\text{-H}}$ = 10.1 Hz, 1H, 4-H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.34 (C-8′), 20.44 (7-CH_3), 22.90, 24.51, 29.46, 29.61, 29.69, 32.09 (C-2′, C-3′, C-4′, C-5′, C-6′ or C-7′), 36.11 (C-1′), 78.06 (C-2), 98.81 (C-4a), 100.34 (C-8), 118.15 (C-4), 120.22 (C-3), 162.63 (C-5), 162.86 (C-7), 165.22 (C-8a); IR (ATR): $\tilde{\nu}$ = 2951, 2921, 2851 cm^{-1} (CH_2, CH_3), 1708 (C=O), 1638, 1553 (C=C), 1448, 1425 (CH_2, CH_3), 1216, 1040 (C–O), 713 (=C–H); UV (CH_3CN): λ_{max}(lg ε) = 228 nm (3.99), 343 (3.76); MS (EI, 70 eV): *m*/*z* (%): 276 (41) [M^+], 261 (1) [M^+-CH_3], 233 (4) [$M^+-C_3H_7$], 219 (7), 177 (10), 163 (100), 121 (33), 85 (15), 43 (73); Anal. Calcd for $C_{17}H_{24}O_3$: C, 73.88; H, 8.75. Found: C, 73.67; H, 8.64.
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14. Human SH-SY5Y neuroblastoma and IPC melanoma cells were cultured in plastic flasks in DMEM-HAM'S F12 medium (GIBCO) containing 10.0% FCS, tetracyclin (10 μg/mL), penicillin (100.0 U/mL), and streptomycin (100.0 μg/mL). For the antiproliferation/cytotoxicity assay cells were seeded on 24-well plates (20,000 cells/well) in a total volume of 1.0 mL per well of the above medium and, prior to the addition of the test compounds, were allowed to grow for 24 h. The cells were then cultured for 7 d with serial dilutions of the test compounds ranging from 50 μg/mL to 1.56 mg/mL. All compounds were added to wells in 10.0 μL volumes of DMSO to give a final DMSO concentration of 1.0% (v/v). The control wells were treated with 10.0 μL DMSO. After a cell culture period of 7 d, the total DNA content of each well was determined using a Fluorimetric CyQuant Cell Proliferation Assay (Molecu-

lar Probes, Eugene, OR). The assay was performed according to the manufacturer's instructions revealing for each well a proliferation index calculated as the quotient of Ex480 and Em520.
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