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Design and Synthesis of Some New Pyranoxanthenone Aminoderivatives with Cytotoxic Activity

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Abstract—The synthesis, DNA binding and in vitro cytotoxicity of a series of novel pyranoxanthones, analogues of the acridone alcaloid acronycine, are described. The new compounds proved to bind weakly to DNA. On the contrary, they exhibited interesting cytotoxic activity against murine leukemia L1210 cell line, as well as against some human solid tumor cell lines.

The acridone alkaloid acronycine (Fig. 1), which was first isolated from *Acronychia baueri* (*Rutaceae*),[1] was found to possess significant in vivo activity against a panel of experimental tumors.[2] However, the clinical development of this promising compound was not successful, partially due to its poor water solubility.[3] Although the exact mode of action of acronycine at both cellular and molecular level has not yet been fully elucidated, the current view is that interaction with DNA, either by intercalation, or by some other non-covalent processes, contributes to the cytotoxicity of this agent in a synergistic manner with other mechanisms of action.[2]

In an effort to develop more efficient analogues, several structural modifications of acronycine have been reported, focusing on substitutions on the acridone chromophore and the pyran moiety as well.[4] A certain number of these derivatives exhibited interesting cytotoxic activity and among them, some proved to be considerably more potent in vivo than acronycine.[5] We have recently reported on the synthesis of some pyranoxanthenones[6,7] and pyranothioxanthenones,[8] which can be viewed as acronycine isosters and demonstrated cytotoxicity comparable, or superior in some cases, to the parent compound. We have also prepared some analogues bearing an aminoalkylamino side chain substitution, which is present in the majority of DNA intercalative cytotoxic agents and have found that this alteration is beneficial and results in an improvement of the activity exhibited by these molecules (compounds I, Fig. 1), as well as by their linear isomers (compounds II, Fig. 1), when tested against the leukemia L1210 cell line.[9]

Prompted by the above results, we performed the synthesis of some structurally related pyranoxanthenone

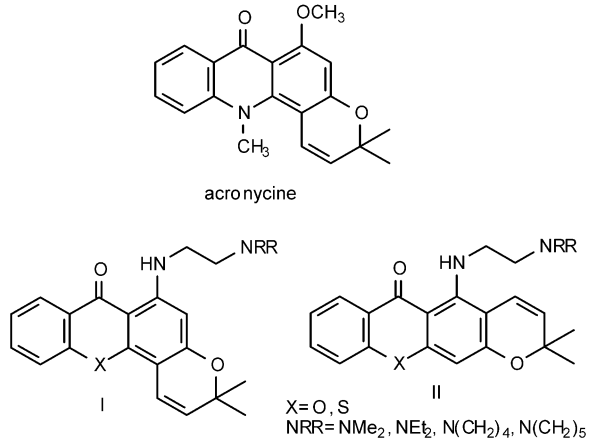

Figure 1. Structure of acronycine and aminosubstituted pyranoxanthenones and pyranothioxanthenones.

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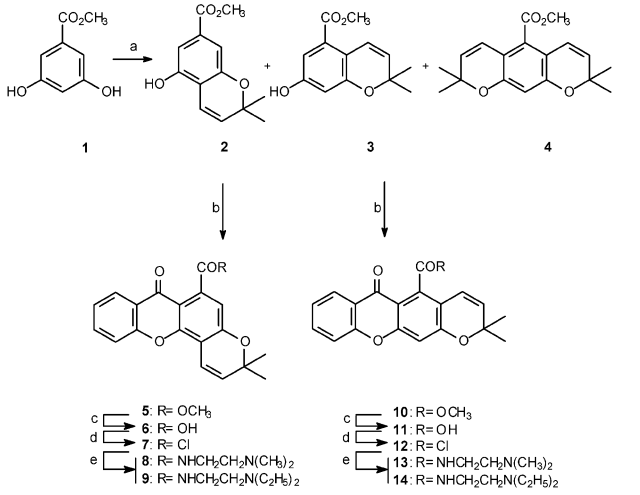
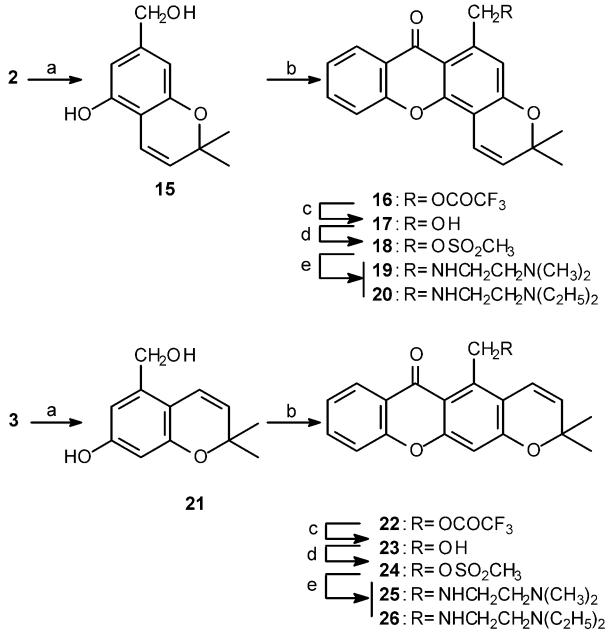


Scheme 1. Synthesis of compounds **8**, **9**, **13**, **14**. Reagents and conditions: (a) K_2CO_3, KI, CuI, 3-chloro-3-methyl-1-butyne, DMF, 75 °C, 24 h; (b) (1) 2-iodobenzoic acid, NaH, TDA-I, CuCl, DMSO, 120 °C, 17 h; (2) $(CF_3CO)_2O$, CH_2Cl_2, rt, 18 h; (c) NaOH 30%, EtOH, reflux, 20 h; (d) $SOCl_2$, 45 °C, 2 h; (e) *N*,*N*-dialkylethylenediamine, Et_3N, toluene, 90 °C, 3 h.



Scheme 2. Synthesis of compounds **19**, **20**, **25**, **26**. Reagents and conditions: (a) $LiAlH_4$, THF, reflux, 3 h; (b) (1) 2-iodobenzoic acid Na salt, NaH, TDA-I, CuCl, THF, reflux, 18 h; (2) $(CF_3CO)_2O$, CH_2Cl_2, rt, 24 h; (c) Na_2CO_3 20%, EtOH, rt, 1 h; (d) CH_3SO_2Cl, Et_3N, THF, rt, 1 h; (e) *N*,*N*-dialkylethylenediamine, Et_3N, THF, reflux, 4 h.

derivatives in an effort to find the optimal structural features required for the biological activity. We have thus inserted a methylene linker between the tetracyclic ring system and the basic side chain, which results in an increased flexibility of the side chain, when compared to compounds I and II, where there is evidence for a strong intramolecular hydrogen bond between the xanthenone carbonyl and the amine hydrogen. On the other hand, we have prepared some derivatives with reduced flexibility of the side chain, by the insertion of an amide link, instead of the methylene, in order to study the effect of this modification in the biological activity.

The synthetic pathway used for the preparation of the amides is outlined in Scheme 1. Methyl 3,5-dihydroxy benzoate (**1**) in DMF, was reacted with 3-chloro-3-methyl-1-butyne, in the presence of cuprous salt as catalyst[10] to provide the corresponding ether, which was not isolated, but was subjected in thermal cyclization at 75 °C, to give, after chromatographic separation, the chromenes **2** (26%) and **3** (40%), together with a small amount (14%) of compound **4**. The structure of these derivatives was confirmed using 1-D and 2-D NMR experiments (HMBC, HMQC). We have clearly observed a J^3 correlation peak between the carbonyl carbon atom and both aromatic protons in the case of **2**. On the other hand, in the case of **3**, we have observed J^3 correlation peaks between the quaternary carbon, which is attached to the ester group, with the olefinic proton and between the carbonyl carbon atom and the aromatic proton. Concerning compound **4**, we have only observed a J^3 correlation peak, between the quaternary carbon and the olefinic protons, while the aromatic proton showed no correlation with the carbonyl carbon atom.

We have also attempted to prepare the chromenes **2** and **3** by reaction of **1** with 3-methyl-2-butenal in the presence of phenylboronic acid and acetic acid, but this approach provided only compound **3** in extremely low yield (3%), together with compound **4** (18%).

The pyranoxanthenones **5** and **10** were derived from 2-iodobenzoic acid and the chromenes **2** and **3** respectively, by copper assisted condensation in the presence of the phase-transfer catalyst tris[2-(2-methoxyethoxy)-ethyl]amine (TDA-I)[11] and subsequent cyclodehydration of the corresponding intermediate 2-phenoxybenzoic acids with trifluoroacetic acid anhydride. The cyclization site of **10** was unambiguously confirmed via 2-D NMR experiments. The methyl esters **5** and **10** underwent basic hydrolysis to form the carboxylic acids **6** and **11** respectively, which were converted to the corresponding acyl chlorides **7** and **12** after treatment with thionyl chloride. Reaction of the above-mentioned chlorides with the appropriately substituted *N*,*N*-dialkylethylenediamines provided the target amides **8**, **9**, **13** and **14**. The amides **13** and **14** were also synthesized via a general method by reaction of the acid **10** with 1,1′-carbonyldiimidazole and the suitable *N*,*N*-dialkylethylenediamine. However, greatly improved yields were achieved by the acyl chloride procedure.

Initial attempts towards the preparation of the diamines **19**, **20**, **25**, **26** (Scheme 2), concerned the reduction of the corresponding amides **8**, **9**, **13** and **14**, after prior protection of the 7- or 6-carbonyl. Unfortunately, the formation of cyclic acetal or thioacetal was not possible, even with the use of the highly reactive 2-chloro-1,3,2-dithiaborolane.[12] Consequently, we used the angular carboxylic acid **6**, which was first converted to the unstable mixed anhydride, after reaction with ethyl chloroformate and then reduced with sodium borohydride in dry THF

with dropwise addition of methanol[13] to give the hydroxymethyl derivative **17**, in 73% yield. However, this method was not successful when it was applied to the linear derivative **11**, probably because of the high instability of the corresponding anhydride, due to steric hindrance. On the contrary, reduction of the acyl chloride **12** with the use of sodium borohydride impregnated on neutral alumina ($NaBH_4$-alox) at room temperature,[14] resulted in the formation of the corresponding hydroxymethyl derivative **23**, in rather low yield (21%). In view of the failure of various methods to reduce specifically and in acceptable yield the carboxyl group of **11**, it was thus decided to develop an alternative synthetic approach for the preparation of both hydroxymethyl analogues **17** and **23**.

The synthetic pathway used is depicted in Scheme 2. Reduction of the chromenes **2** and **3** with $LiAlH_4$ gave the corresponding alcohols **15** and **21**. Copper assisted condensation of these analogues with 2-iodobenzoic acid sodium salt and subsequent cyclodehydration in the presence of trifluoroacetic acid anhydride, yielded the trifluoroacetates **16** and **22**, respectively. The trifluoroacetates were easily hydrolyzed to the corresponding hydroxymethyl derivatives **17** and **23**, which were then converted to the mesylates **18** and **24**, respectively. The mesylates were not isolated, but the leaving group was readily displaced with the appropriately substituted secondary amines to provide the target derivatives **19**, **20**, **25** and **26** in reasonable yields (78–83%).

In order to examine the DNA-binding properties and the in vitro antineoplastic activity of these agents, the free base forms of the amines **19**, **20**, **25** and **26** were converted into their water-soluble hydrochloride or fumarate addition salts, by treatment with either hydrochloric or fumaric acid, respectively, in ethanol. Concerning the amides **8**, **9**, **13** and **14**, the evaluation was made to the free bases, since their hydrochloric, fumaric or maleic salts were highly hydroscopic.

The new compounds were assayed in vitro for their DNA-binding affinity in calf thymus DNA, using an ethidium bromide displacement assay.[15] Results were expressed as EC_{50}, the concentration of the compound that causes a 50% reduction in the fluorescence of the calf thymus DNA/ethidium bromide complex. Cytotoxic activity was assayed in the established model of the murine leukemia cell line L1210, as well as, against two human solid tumor cell lines (colorectal adenocarcinoma HT-29 and lung carcinoma A549), using a modification of the MTT assay[16] after exposure to the test compounds for 48 h. Results were expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls. Cell-cycle analysis was performed on a FACSCalibur (Becton Dickinson) flow cytometer using the ModFit software, after trypsinization of the treated cultures, fixation in 50% ethanol, and staining with an RNAse-containing propidium iodide solution.[5] The results, including the data for the reference compounds, acronycine and mitoxantrone, are shown in Tables 1 and 2.

Table 1. Inhibition of proliferation (IC_{50} values in μM) and DNA binding (EC_{50} values in μM) of target derivatives

	IC_{50} (μM)[c]			DNA binding, EC_{50} (μM)[d]
	Lung A549	Colon HT29	L1210	
8	90 (±18)	12 (±2)	16 (±1)	197
9	60 (±7)	30 (±2)	10 (±0.3)	150
13	57 (±11)	60 (±10)	12 (±1)	107
14	51 (±4)	40 (±4)	9 (±1.1)	100
19[a]	44 (±7)	8.8 (±1.3)	4.1 (±0.3)	74
20[b]	11 (±2)	1.7 (±0.3)	2 (±0.3)	42
25[a]	25 (±3)	6.4 (±0.4)	4 (±0.1)	39
26[a]	8 (±1.1)	2 (±0.1)	2 (±0.1)	30
Mx	0.3 (±0.04)	8 (±0.5)	0.04 (±0.005)	0.4
Ac	>100	>100	27 (±3)	82

Reference compounds: Mitoxantrone (Mx) and Acronycine (Ac).
[a]Hydrochloride.
[b]Fumarate.
[c]Values represent the mean of three experiments (standard deviation is given in parentheses).
[d]Values represent the mean of two experiments (±1–10%).

Table 2. Cell-cycle-phase distribution of HT-29 cell-cultures exposed to target derivatives (10 μM) for 22 h[a]

	G_0/G_1	S	G_2/M
Untreated	59.13	29.07	11.81
14	61.24	1.06	37.70
20[b]	79.70	12.53	7.77
26[c]	69.92	21.22	8.86

[a]One out of two similar experiments is depicted.
[b]Fumarate.
[c]Hydrochloride.

Table 3. IC_{50} values (μM) and DNA binding of previously reported analogues I and II

	IC_{50} (μM)[d]			DNA binding, EC_{50} (μM)[e]
	Lung A549	Colon HT29	L1210	
I,[a] X=O, R=Me	23 (±8)	8 (±1)	7.1 (±0.8)	89
I,[b] X=O, R=Et	>100	42 (±7)	8.4 (±1.1)	88
II,[a] X=O, R=Me	30 (±4)	23 (±2)	5.8 (±0.6)	ND[c]
II,[a] X=O, R=Et	25 (±1)	8 (±0.9)	5.1 (±0.2)	ND[c]
Mx	0.3 (±0.04)	8 (±0.5)	0.04 (±0.005)	0.4
Ac	>100	>100	27 (±3)	82

Reference compounds: Mitoxantrone (Mx) and Acronycine (Ac).
[a]Hydrochloride.
[b]Fumarate.
[c]ND, not detected.
[d]Values represent the mean of three experiments (standard deviation is given in parentheses).
[e]Values represent the mean of two experiments (±1–10%).

In general, all the new derivatives were more potent than acronycine in all the cell lines tested. The cytotoxic potency of the diamines **19**, **20**, **25** and **26** is clearly higher than that of the corresponding carboxamides **8**, **9**, **13** and **14** in the three cell lines. The above-mentioned diamines are 6–13-fold more potent than acronycine

against the L1210 cell line, possess a weak activity against the Lung A549 cell line, being clearly less efficient than the reference drug mitoxantrone, but the data are more profound in the case of the colon adenocarcinoma cell line, where they possess IC_{50} values equal, or up to 4 times lower than mitoxantrone. The DNA binding data show that the new derivatives possess weak ethidium bromide displacement potency, but again the diamines bind better than the carboxamides and acronycine. It should also be noted that the linear compounds (**25** and **26**) are better binders than their angular isomers (**19** and **20**). Compounds **20** and **26**, which both bear a diethylaminoethylamino substitution are the most potent derivatives in this series and exhibit a universal profile of IC_{50} values (1.7–11 μM) for the whole spectrum of cell lines used. DNA content analysis of cultures treated with some of the derivatives (Table 2) has shown, that the carboxamide **14** (even at relatively low concentration comparing to its IC_{50}) induces a partial accumulation in the G_2/M phase, as shown previously also for acronycine,[5] while the diamines **20** and **26** are blocking the S phase, in agreement with their DNA-binding potencies. A marked subdiploid peak observed after exposure to compound **20** and — to a lesser extent—**14** indicated cell death due to apoptosis.

Cytotoxicity comparison of the new derivatives can be made with parallel results, obtained with the previously reported 6-dialkylaminoethylamino pyrano[2,3-*c*]xanthen-7-ones (I, X = O, R = Me or Et) and 5-dialkylaminoethylamino pyrano[3,2-*b*]xanthen-6-ones (II, X = O, R = Me or Et), through the data presented in Table 3.

The results indicate that the compounds synthesized in the present study possess an improved biological profile against murine leukemia as well as against the solid tumor cell lines tested, providing evidence that the side chain elongation is favorable for the expression of cytotoxicity of this class of compounds.

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