EFFICIENT ONE-POT SYNTHESIS AND EVALUATION OF THE CYTOTOXIC ACTIVITY OF 2,4-DIMETHYL-1*H*-CHROMENO[4',3':4,5] PYRIDO[2,3-D]PYRIMIDINE-1,3,7(2*H*,4*H*)-TRIONE

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Abstract: A 2,4-dimethyl-1*H*-chromeno[4',3':4,5]pyrido[2,3-d]pyrimidine-1,3,7(2*H*,4*H*)-trione **3** was synthesized via a one-pot procedure. Its structure was established on the basis of FTIR, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, MS data and elemental analysis. The new compound was assayed for antiproliferative/cytotoxic effects in a panel of human tumor cell lines after 72 h exposure. MTT-dye reduction assay was used as a read-out system and IC_{50} as an end-point to evaluate efficacy. Our cytotoxicity screening indicates that the presented fused system exhibits moderate antiproliferative effect in a panel of human tumor cell lines of different origin and cellular type.

Key words: antiproliferative/cytotoxic effects, coumarin, pyrido[2,3-d]pyrimidine, MTT-assay, uracil

Introduction

The pyrimidine system is an important pharmacophore [1, 2] interfering in the synthesis and function of nucleic acids, e.g. cytostaticum 5-Fluorouracil and the HIV/AIDS drugs as Zidovudine, Zalcitabine, Zonavir. Also, pyrido[2,3-d]pyrimidines are very useful class of annulated uracils with biological significance due to their relation to the purine and pteridine systems. Those fused heterocycles and their derivatives are very efficient as cardiotonic [3], antiallergic [4], antihypertensive [5], anticancer [6, 7] and antitubercular [8] agents. Therefore, the synthesis of pyrido[2,3-d]pyrimidines has been of remarkable interest in the preparation of such complex macromolecules, namely in synthetic manipulations of uracils [9]. The synthetic strategies of Broom *et al*. [10], Wamhoff *et al*. [11], Hirota *et al.*[12], and Mohit L Deb *et al.* [13] involve employing multistep two-component reactions. For the first time M.Gohain *et al*. [14] suggested an unprecedented one-pot synthesis of novel pyrimido[4,5-d]pyrimidines and pyrido[2,3-d]pyrimidines, respectively, in refluxing DMF/nitrobenzene achieving good yields in 3–4 hours. Being essentially a gem-activated alkene with a good leaving group (chlorine atom) 4-chlorocoumarin-3-carbaldehyde **1** was used for the generation of an array of fused nitrogen heterocycles [15]. It is known that 4-chlorocoumarin-3-carbaldehyde **1** exhibits a variety of properties and can react with substituted anilines [16], amidines [17], arylisocyanide [18], o-hydroxyacetophenone [19], benzylamines [20], sodium azide [21], aryl aldehydes [22], ethyl cyanoacetate [23], ethyl 3-aminocrotonate [24], 1,3-bis(trimethylsilyloxy)-1,3-butadienes [25], Wittig phosphoranes [26] and arylhydrazines. [27]. Having an electron withdrawing formyl group 4-chlorocoumarin-3-carbaldehyde **1** undergoes conjugated addition/elimination reactions with dinucleophiles at the 4th position of the coumarin system, followed by intramolecular cyclization via electrophilic formyl function to coumarins 3,4-fused to pyrazoles, isoxazoles, pyrimidines, pyrroles, pyridines, quinolines, etc. heterocycles.

The biological and industrial importance of coumarins has led to a considerable amount of synthetic work in the field of coumarins possessing 3,4-carbocyclic- and 3,4-heterocyclic fused ring systems. Much attention has been paid to the tumor-inhibiting and cytotoxic properties of coumarins, their derivatives and structural analogs [28]. Thus, it is well appreciated that the cytotoxic coumarins are a feasible source of new antineoplastic leads, which by virtue

of the pleiotropic modes of action (incl. antimitotic, antiangiogenic, proapoptotic etc.) might also help addressing the issues of side effects, toxicity and the emerging multi-drug resistance.

We report here on a simple and efficient synthesis in quantitative yields of fused pyrido[2,3-d]pyrimidine with a coumarin moiety from 4-chlorocoumarin-3-carbaldehyde **1** and 6-amino-1,3-dimethyluracil **2** in acetic acid**,** as well as on the assessment of its antiproliferative potential in a panel of human tumor cell lines, representative for some important types of human neoplastic diseases.

Materials and methods

General

Melting points were determined in a capillary tube on a Buchi 535 apparatus. The FTIR spectra were recorded on a Nicolet IS10 FT-IR spectrometer from Thermo Scientific (USA) using ATR technique. The NMR spectra were recorded on a Bruker Avance II+ 600 (600.13 for ^{1}H MHz and 150.92 MHz for ^{13}C NMR) with TMS as internal standards for chemical shifts (d, ppm). The assignment of the ^{1}H and ^{13}C NMR spectra was made on the basis of DEPT, COSY and HSQC experiments. Elemental analyses were performed by Microanalytical Service Laboratory of Faculty of Pharmacy, Medical University of Sofia, using Vario EL 3 CHNS(O). Mass spectra were measured on a ESI (HESI-II) HR-ESI-MS Model: Q Exactive Plus (Thermo Scientific, Bremen, Germany) by Service Laboratory of Faculty of Pharmacy, Medical University of Sofia. The purity of the new compound was checked by TLC on silica gel 60 GF 254 Merck pre-coated aluminum sheets. Commercially available solvents for the reactions and TLC were used after distillation (dried when needed). Reagents (6-amino-1,3-dimethyluracil and 4-hydroxycoumarin) were commercial grade and used without further purification. Formic acid, 2-propanol, L-glutamine were purchased from AppliChem GmbH, Darmstadt, Germany. Fetal calf serum (FCS) and RPMI 1640 medium were purchased from Sigma–Aldrich Co., the USA. The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied from Merck Co., Germany. The referent antineoplastic drug melphalan was used as a commercially available pharmaceutical grade substance.

Synthesis of 4-Chlorocoumarin-3-carbaldehyde (1)

It was prepared by a previously reported procedure [29] as a white solid, yield 84%, m.p 120-122 °C (lit. m.p 120-122 °C). The structure of the compound was proved by FTIR, ^{1}H NMR, ^{13}C NMR. The data was in accordance with the literature.

Synthesis of 2,4-dimethyl-1*H*-chromeno[4‘,3‘:4,5]pyrido[2,3-d]pyrimidine-1,3,7(2*H*,4*H*)-trione (3). Another name is 2,4-dimethyl-4*H*-8-oxo-2,4,5-triazo-benzo[c]phenanthrene-1,3,7-trione

4-Chlorocoumarin-3-carbaldehyde **1** (1.043 g, 5 mmol) was dissolved in 20 ml of glacial acetic acid under vigorous stirring at room temperature. 6-Amino-1,3-dimethyluracil **2** (0.776 g, 5 mmol) was added to the solution (Scheme 1). After 15 min the color of the reaction mixture changed from light yellow to brown and then light yellow crystals precipitated from the reaction mixture. The product was monitored using TLC. After 60 min the crystals were filtered off, washed with ethanol (10 ml) and H_2O (5x10 ml). The product was collected and recrystallized from ethanol. Yield 1.411g (91 %), m.p. 325-327 °C. FTIR (ATR) ν 3098, 2970, 1716, 1660, 1620, 1601, 1587, 1568, 1557 cm^{-1}. ^{1}H NMR (600 MHz, pyridine-d_5): 3.45 [s, 3H, (N-2)-CH_3], 3.72 [s, 3H, (N-4)-CH_3], 7.37 (ddd, *J* 7.8, 7.2, 0.9 Hz, 1H, 11-H), 7.58 (d, *J* 8.3 Hz, 1H, 9-H), 7.71 (ddd, *J* 8.3, 7.2, 1.2 Hz, 1H, 10-H), 8.36 (dd, *J* 7.8, 1.7 Hz, 1H, 12-H), 9.55 (s, 1H, 6-H). ^{13}C NMR (150 MHz, pyridine-d_5): 28.2 [(N-2)-CH_3], 29.8 [(N-4)-CH_3], 109.6 (C-12c), 112.6 (C-6a), 118.4 (C-9), 121.9 (C-12a), 122.9 (C-12b), 125.3 (C-11), 126.6 (C- 12), 135.8 (C-10), 139.6 (C-6), 151.0 (NH-CO-NH), 153.6 (C-4a), 155.4 (C-8a), 159.9 (COO), 162.3 (NH-CO). HRMS-ESI *m/z*: $[M+H]^+$ calcd for $C_{16}H_{11}N_3O_4$, 310.08164; found, 310.08223. Anal calcd for $C_{16}H_{11}N_3O_4$: C 62.14, H 3.58, N 13.59. Found: C 62.31, H 3.50, N 13.51.

Cell lines and culture conditions

The study was conducted using a panel of cell lines: SKW-3 (ACC 53) – human T-cell leukemia, originally described to be established from the peripheral blood of a 61-year-old man with T cell chronic lymphocytic leukemia (CLL) in 1977; however, DNA fingerprinting and cytogenetic analysis showed cross-contamination with cell line KE-37; KE-37 was established from a 27-year-old man with acute lymphoblastic leukemia (ALL) in 1979; HL-60 (ACC 3) - human acute myeloid leukemia (AML), established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia (AML FAB M2) in 1976; cells are apparently tetraploid derivates of the hypodiploid original; K-562 (ACC 10) – human chronic myeloid leukemia (CML) es-

tablished from the pleural effusion of a 53-year-old woman with CML in blast crisis in 1970; cells carry the BCR-ABL1 e14-a2 (b3-a2) fusion gene; the used subclone weakly expresses MHC class I (image); MDA-MB-231 (ACC 732) – human adenocarcinoma, established in 1973 from the pleural effusion of a 51 years old Caucasian woman with metastatic mammary carcinoma; cells were described of being negative for cytoplasmic estrogen receptors.

The cell lines were purchased from the DSMZ GmbH, (Braunschweig, Germany). They were cultured under standard conditions - RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 2 mM L-glutamine, in cell culture flasks, housed at 37 °C in an incubator ‚BB16-Function Line' Heraeus (Kendro, Germany) with humidified atmosphere and 5 % of CO_2. The cell cultures were maintained in log phase by supplementation with fresh medium two or three times weekly.

Cytotoxicity assessment (MTT-dye reduction assay)

The assay was carried out as described elsewhere [30] with minor modifications [31]. The cellular survival rates after treatment with the tested compounds was assessed using the MTT-dye reduction assay, based on the metabolic reduction of the yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide to a violet formazan product via the mitochondrial succinate dehydrogenase in viable cells. Exponentially growing cells were plated in 96-well flat-bottomed microplates (100 µl/well) at a density of $1\square10^5$ cells per ml. After a 24 h incubation period at 37 °C they were treated with graded concentrations of the extract (or its fractions) for 72 h. For each concentration a set of 8 wells was used. After the exposure period 10 µl MTT solution (10mg/ml in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37 °C and the MTT-formazan crystals formed were dissolved through addition of 100 µl/ well 5% of formic acid in 2-propanol. The MTT-formazan absorption was determined using a microprocessor controlled microplate reader (Labexim LMR-1) at 580 nm.

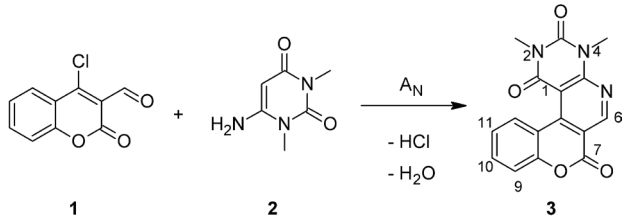
Bioassay data processing and statistics

The MTT-bioassay data were normalized as percentage of the untreated control (set as 100 % viability), were fitted to sigmoidal dose response curves and the corresponding IC_{50} values (concentrations causing 50 % suppression of cellular viability) were calculated using non-linear regression analysis (GraphPad Prizm Software for PC). The statistical processing of biological data included the Student's t-test whereby values of $p \leq 0.05$ were considered as statistically significant.

Results and Discussion

Chemistry

Our synthetic strategy utilizing 4-chlorocoumarin-3-carbaldehyde **1** and 6-Amino-1,3-dimethyluracil **2** in glacial acetic acid (20 ml) at r.t. affords an unprecedented one-pot synthesis of novel tetracyclic product **3** (Scheme 1) that combines into a single molecule two well established in medical chemistry pharmacophoric moieties - coumarin and uracil. The product **3** was obtained in good yield and excellent purity. The molecular architecture of the tetracyclic product **3** was established by means of NMR, FTIR, MS spectra, elemental analysis and melting point. The precise assignment of ^{1}H NMR and ^{13}C NMR spectra was accomplished by measuring 2D homonuclear correlation (COSY), DEPT-135 and 2D inverse detected heteronuclear (C–H) correlations (HMQC and HMBC).



Scheme 1. Synthesis of 2,4-dimethyl-1H-chromeno[4',3':4,5]pyrido[2,3-d]pyrimidine-1,3,7(2H,4H)-trione 3.

First of all, there are no broadened signals for exchangeable protons from the amino group of the substrate aminouracil **2** in the ^{1}H-NMR spectra (Figure 1). That means the primary amino group has participated in the cyclization. There are four aromatic protons from the coumarin moiety. The two N-methyl groups from the uracil moiety give two sharp singlets at δ=3.45 ppm and 3.72 ppm, while the proton at C-6 gives a singlet at δ=9.55 ppm. That value is close to the one for the proton at δ-position of the pyridine ring (δ=8.593 ppm), but it is deshielded because of the two carbonyl groups conjugated to the pyridine ring. All signals expected for the carbon atoms according to the molecular formula are available in ^{13}C-NMR spectrum. The signals assignment is described in details in the *Materials and methods Part*. The ion observed by mass spectrometry is $[M+H]^+$ (Figure 2.).

The suggested mechanism of this cyclization (Scheme 2) in fact consists of a convergent synthesis of condensed tetracyclic system **3** of pyrimidine and coumarine moieties including the following steps:

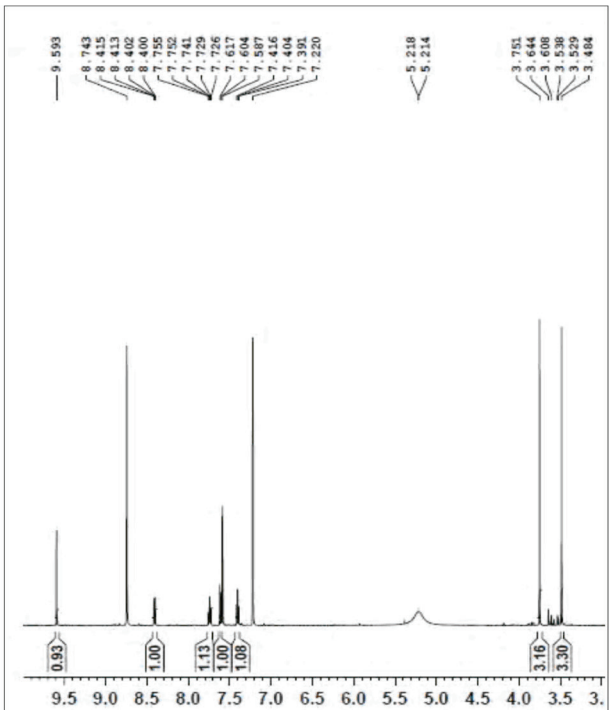


Figure 1. ^{1}H NMR spectrum for compound 3 in pyridine-d_5.

The first step is the nucleophilic addition of the 6-aminouracil **2** to the chloroaldehyde **1** accompanied by elimination of HCl. The nucleophilic addition of the C-5 of uracil reactant to C-4 of coumarin substrate was achieved because of the low electron density at 4th position due to the strong electron-withdrawing inductive effect of chlorine as well as to the two carbonyl groups conjugated by a double bond (C-3)=(C-4). That makes 4-chlorocoumarin-3-carbaldehyde **1** a highly reactive Michael receptor. It is known that, C-5 of uracil **2** has enhanced nucleophilicity due to the electrondonating effect (+M) of the two nitrogen atoms conjugated by a (C-5)=(C-6) double bond. Therefore, the Michael-type nucleophilic addition of uracil **2** to 4-chlorocoumarin-3-carbaldehyde **1** could be easily formulated (Intermediate **A**).

The second step was realize through the intramolecular Schiff base formation between the aldehyde group of the coumarin moiety (with a favourable spatial location) and the amino group at the C-6 position in the uracil moiety (Intermediate **B**).

Additionally, we carried out an alternative experiment with equimolar amount of 4-chlorocoumarin-3-carbaldehyde **1** and 6-amino-1,3-dimethyluracil **2** in 20 ml abs. ethanol and the resulted solution was refluxed for 6 h. After 15 min the colour of the re-

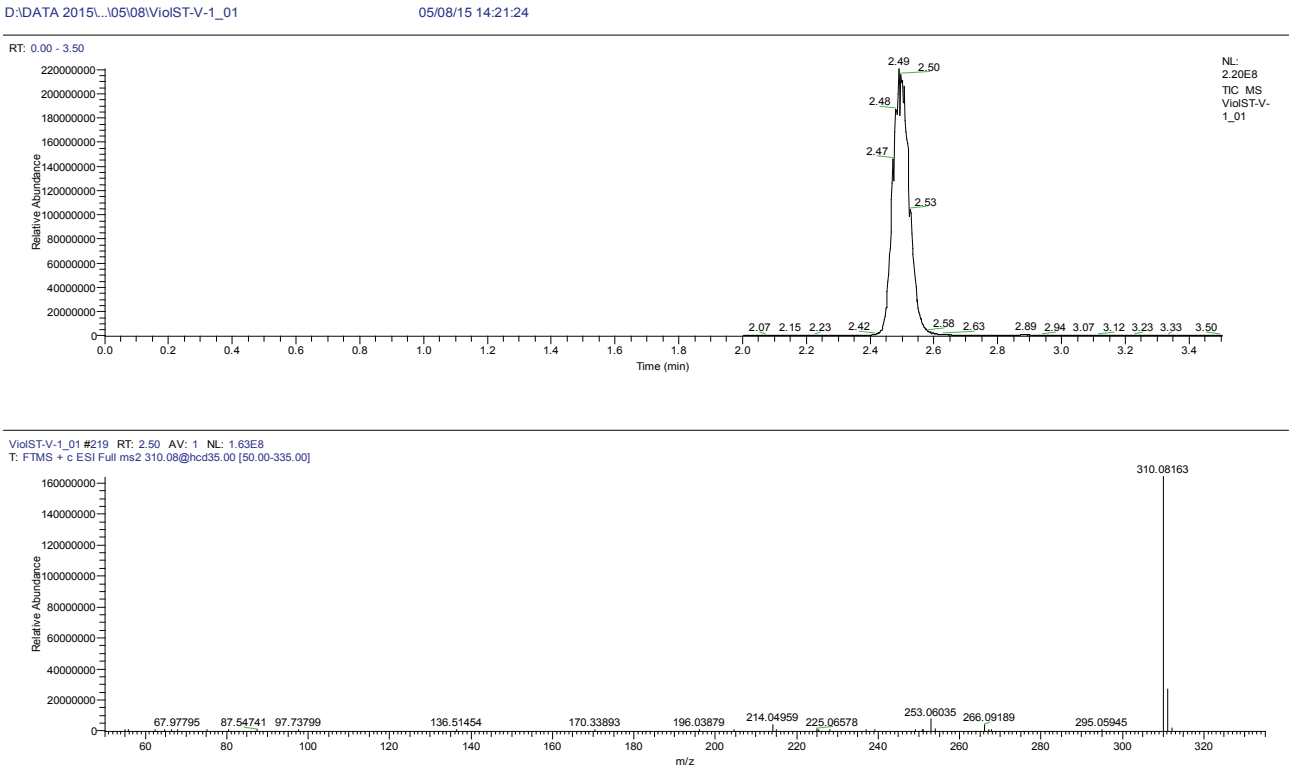
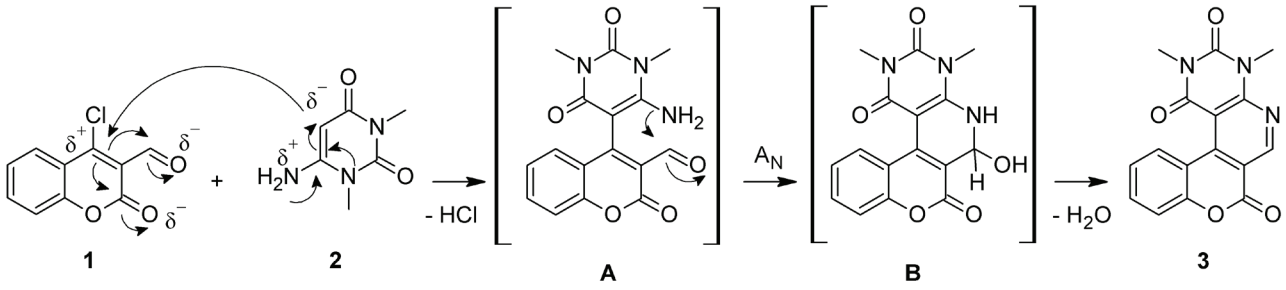


Figure 2. MS-spectrum (HRMS-ESI) for compound 3.



Scheme 2. Reaction mechanism for the synthetic route for compound 3.

action mixture changed from light yellow to red-brown, then yellow crystals precipitated from the reaction mixture. After 6 h the crystals were filtered off, washed with H_2O, collected and dried in air. The product was recrystallized from ethanol. The yield 1.16 g (60 %) was lower than the one obtained according to the main procedure, described above.

The results have demonstrated that heteroannulation on the double bond of uracil is possible under simple and mild conditions using suitable organic substrates.

Cytotoxic and proapoptotic effects

This study was conducted in an attempt to evaluate the anti-cancer potential of a series of 4-aminocoumarins [32]. The antiproliferative/cytotoxic effect of the tested compound was assessed using the standard MTT-dye reduction assay in a panel of human tumor cell lines representative for some important neoplastic diseases. The panel consisted of the acute lymphoid leukemia derived KE-37 (SKW-3), the acute myeloid leukemia derived HL-60, the chronic myeloid leukemia derived K-562, and the breast cancer derived MDA-MB-231. The tested compound exerted concentration-dependent cytotoxicity, which allowed the construction of sigmoidal dose response curves and calculation of the equieffective IC_{50} concentrations (the concentration leading to 50 % reduction of the cellular viability) which served as criteria for juxtaposition of the antiproliferative/cytotoxic activity. Throughout the onco-pharmacological survey the clinically applied alkylating agent melphalan was employed as a positive control. The respective IC_{50} concentrations were calculated via nonlinear regression analysis and summarized in Table 1.

Evident from the equieffective half-maximal inhibitory concentrations (IC_{50}), the tested compound exerted cytotoxic properties. The chemosensitivity of the individual cell lines varied a little but generally the K-562 and KE-37 (SKW-3) were more responsive than HL-60 and MDA-MB-231. In the latter cell lines, however, the cytotoxicity of the compound was encsountered at concentrations greatly exceeding 100 μM.

Conclusion

The simple, mild and efficient method for the synthesis of a novel 3,4-fused coumarin with pyrido[2,3-d]pyrimidine has been described. The spectroscopic data confirm the formation of the target compound **3** *via* cyclization between 4-chlorocoumarin-3-carbaldehyde **1** and 6-aminouracil **2** which occurs through 4-Cl from coumarin and C-5H from aminou-

Table 1. *MTT-bioassay data for the 2,4-dimethyl-1H-chromeno[4',3':4,5]pyrido[2,3-d]pyrimidine-1,3,7(2H,4H)-trione 3 against a panel of human tumor cell lines.*

Cell lines		**IC_{50} (μM)[2]**	**Contr. Melphalan**
HL-60	acute myeloid leukemia derived HL-60,	291.7 ± 3.2	11.2 ± 1.9
SKW-3	human T-cell leukemia[3]	273.3 ± 5.9	14.6 ± 3.1
K-562	chronic myeloid leukemia	274.2 ± 6.9	28.3 ± 3.2
MDA-MB-231	ER-negative breast cancer.	> 400.0	13.7 ± 1.9

[1]Synthesized as described elsewhere; [2]means ± sd from eight independent experiments; [3]SKW-3 is a KE-37 derivative.

racil followed by 6-NH_2 and HC=O addition/elimination reaction. Our cytotoxicity screening indicates that the presented fused system exhibits moderate antiproliferative effect in a panel of human tumor cell lines of different origin and cellular type using the MTT-dye reduction assay as a read-out system and the IC_{50} end-point. The established cytotoxic activity, albeit moderate, supports the well-established antineoplastic potential of structurally diverse natural and synthetic coumarins.

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