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Biotransformation of the flavonoid tiliroside to 7-methylether tiliroside: bioactivity of this metabolite and of its acetylated derivative

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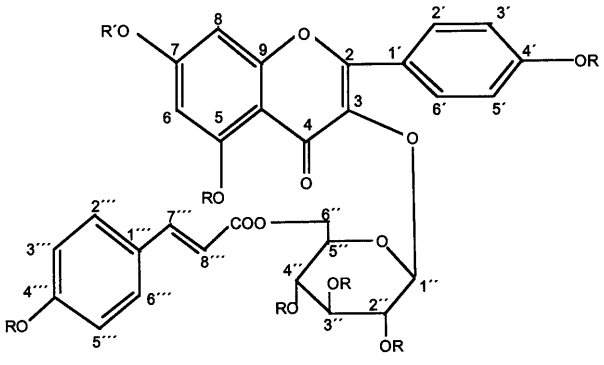
Abstract. Incubation of kaempferol-3-O-β-D-(6″-E-p-coumaroyl)-glucopyranoside (tiliroside) (**1**) with *Aspergillus nidulans* gives the 7-methyl ether of tiliroside (**2**) which is a new compound. Its structure is determined by spectroscopic methods. Cytotoxic studies of **2** and of its acetylated derivative **2a** were carried out in vitro against fourteen human leukemic cell lines. Results clearly show that compound **2** is ineffective against all leukemic cell lines tested. On the contrary, compound **2a** exhibited cytotoxic activity against four of the cell lines (HL60, DAUDI, HUT78 and MOLT3) and additionally, a dose- and time-dependent effect on DNA synthesis.
Key words. *Aspergillus nidulans*; tiliroside; kaempferol glycosides; biotransformation; cytotoxicity; DNA synthesis.

Biotransformations are useful tools for the production of medicinal chemicals from natural products. Such a product is kaempferol-3-O-β-D-(6″-E-p-coumaroyl)-glucopyranoside (tiliroside) (**1**), which has been isolated from natural sources [1–3]. Flavonoids are known to exhibit a broad spectrum of pharmacological activities, including antitumor, antimicrobial, antiviral, central vascular system and enzyme inhibiting activities [4]. Our knowledge of the microbial transformation of flavonoids, especially flavonoid glycosides, is very limited at present. *Aspergillus niger* has been used successfully to transform in vivo flavone, flavanone and isoflavone [5, 6]. Isolates of the genus of Aspergilli are capable of producing extracellular enzymes that degrade flavonols [7]. Compound **1** is known to be an inactive product against a panel of human cell lines [1, 8]. Some metabolites may be more active than their parent compounds [9], and our work examines the biotransformation of **1** by *A. nidulans* in order to study the cytotoxicity of its metabolites. Thus, we report in this paper the structure of the metabolic product 7-methylether tiliroside (**2**) as well as its cytotoxic activity and effect on DNA synthesis together with its acetylated derivative **2a**.

Materials and methods

Microorganism and culture conditions. A prototrophic strain of *A. nidulans* [10] was used throughout this work. The fungus was grown at 37 °C under continuous shaking conditions (200 rpm). A chemically defined medium containing : sucrose (2 g) , $NaNO_3$ (2 g), $MgSO_4\ 7H_2O$ (1 g), KH_2PO_4 (1 g), KCl (0.5 g), $FeCl_3$ (0.01 g) in distilled H_2O (1000 ml) served as minimal medium (MM). Complete medium (CM) was prepared by addition of yeast extract (15 g), malt extract (15 g) and bacteriological peptone (15 g) per 1000 ml of MM. The ability of *A. nidulans* to metabolize substance **1** was examined in initial screening tests. A series of conical flasks containing either CM or MM with the addition of 0.25, 0.5, 0.75, 1, 1.5 and 3 mg/ml of **1**, were inoculated with identical inocula of *A. nidulans* spores and optimal experimental conditions (MM + 1.5 mg/ml of **1**) were established. A time course of the consumption of **1** and its biotransformation into new substances was followed by collecting the content of two independent flasks (from a series of flasks), at 3 h intervals, until the cultures had passed late exponential phase (i.e. several hours in the stationary phase). The samples were centrifuged and the resulting supernatant was condensed by lyophilization. TLC (silica gel) was used as an analytical method. UV-Vis was used as a method for percentage determination of the uptake of compound **1**.



1 R=R′=H
1a R=R′=COCH$_3$
2 R=H R′=CH$_3$
2a R=COCH$_3$ R′=CH$_3$

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The resulting mycelial pellets were washed three times in Ringer's solution, broken by sonication and their cytosolic content including **1** and new substances was extracted with MeOH. The purity of the compounds were examined by TLC on silica gel (Kieselgel 60 F_{254} Merck) with CH_2Cl_2-MeOH (99:1–90:10). Stock solutions of **1** were made up in DMF. To determine the nature of new substances prepared from **1**, larger volume cultures were employed in flasks containing a total of 800 mg of **1**.

Isolation of 2. The mycelial pellets of *A. nidulans* were extracted with MeOH. The residue (600 mg) was chromatographed over 20 g silica gel, using CH_2Cl_2 and CH_2Cl_2-MeOH mixtures of increasing polarity followed by pure MeOH. A total of 200 fractions were obtained. Fractions 160–185 ($\times$20 ml) were examined by HPLC with a gradient system consisting of two LKB2248 pumps. Compound **2** was detected with a single wavelength absorbance detector at 311 nm and was injected on a Techsil 10C18 column (25 cm $\times$ 7.8 mm). Mixtures of MeOH-AcOH 5% from 40% to 50% in 20 min were used as eluent at a flow rate of 4 ml/min. 7-methylether tiliroside (**2**) was obtained as yellow powder (24 mg). Compound **2** was dissolved in Ac_2O-Py and left for 48 h at room temperature to yield **2a**.

^{1}H- and ^{13}C-NMR spectra were recorded on a Brucker HX 200 spectrometer operating at 200 and 50 MHz respectively; UV spectra were taken on UV-160A Shimadzu spectrometer; MS were recorded with a Nermag R 1010C spectrometer. Thioglycerol was used as a matrix.

Cytotoxic activity. Compound **1** is known to be an inactive product against a panel of human and murine cell lines [8]. Thus for reasons of comparison we examined the metabolic compound **2** and its acetylated derivative **2a** for cytotoxic activity on leukemic cells. The following cell lines have been used: CCRF-CEM [11, 12], MOLT3 [13], JURKAT [14], HUT78 [15], H9 [16] , (T cells) KM3 [17], NAMALWA [18], DAUDI [19, 20], SDK [21], JIYOYE [22] , CCRF-SB [23] (B cell lines), HL60 [24] (promyelocytic cell line), K562 [25] (proerythrocytes), U937 [26] (monocytes). All cell lines were grown as exponentially proliferating suspension cultures in RPMI-1640 medium (Gibco Europe Ltd, Scotland), supplemented with 10% heat inactivated fetal calf serum (Myoclone Gibco), 2 mM L-glutamine (Gibco), and 50 μg/ml gentamycin and incubated at 37 °C, in a humidified atmosphere and 5% CO_2. Viability was assessed by trypan blue dye exclusion and was always greater than 98%. Peripheral blood mononuclear leukocytes (PBML) were obtained from the peripheral blood of normal volunteers after Ficoll-Hypaque centrifugation [27]. The compounds to be tested were dissolved in DMSO at the appropriate concentrations and stored at -40 °C. To determine their cytotoxic activity, the compounds were added at the same time to each cell line or PBML (1×10^6 cells/ml final cell density). An equivalent amount of DMSO was added to control cultures. After the addition of the flavonoids the cells were cultured in microplates for 48 hours in a moist atmosphere of 5% CO_2 in air. Cytotoxicity was determined by the MTT method [28, 29]. Four hours before the end of the 48 h incubation period, MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide, Sigma) dissolved in PBS (phosphate buffered saline) at 5 mg/ml, was added in the cell cultures at a final concentration of 50 μg/ml. At the end of the 48 h incubation period, acid isopropanol was added to the wells and the optical density was measured with an ANTHOS HT II Microelisa reader, using a test wavelength of 550 nm. Using this method the lethal dose 50 (LD_{50}) (i.e. the concentration that provoked a 50% death rate at the cell population compared to the control) was determined. Data were analysed using a two-tailed Student's *t*-test.

Measurements of DNA synthesis. DNA synthesis was assayed at 1, 4, 24 and 48 h after the addition of the compound to be tested. The cells were incubated for 1 h, with 10 μCi of [^{3}H]thymidine (Amersham, U.K.), before the end of each interval under the same conditions as for the MTT assay. At the end of the incubation period the cells were harvested in an automatic cell harvester and the amount of radioactivity incorporated into macromolecules was measured in a liquid scintillation counter (Packard IL) and expressed as counts per minute (CPM). The compound **2a** was tested at three concentrations 2, 10 and 20 μg/ml. Doses higher than 20 μg/ml were found to be significantly cytotoxic for resting PBML. Therefore 20 μg/ml of **2a** was the highest concentration examined in this kind of experiment.

Results and discussion

Identification of compound 2. Compound **2** had a molecular formula $C_{31}H_{28}O_{13}$. The UV (MeOH) spectrum showed absorption maxima at 268 and 315 nm. On addition of base (NaOAc), no bathochromic shift was observed in contrast to **1**, suggesting that it is indeed a 7-hydroxyl derivative of flavonoid. The ^{1}H- and ^{13}C-NMR chemical shifts of **2** (tables 1 and 2) were assigned on the basis of H-H and C-H shift correlation NMR spectral analysis. The spectral data of **2** are very similar to those of **1** [2, 3] and related compounds [30]. The presence of an additional methoxy signal (δ 3.80) showed that compound **2** is a methylether of **1**. This was further substantiated by long range H-H shift correlation between the OMe (δ 3.80) and the H-6 (δ 6.20) and H-8 (δ 6.42) of **2**.

Compound **1**, which has been isolated previously by us [1], was dissolved in Ac_2O-Py and left for 48 h at room temperature to yield **1a**.

The ^{1}H-NMR signals H-6 and H-8 of **2a** showed upfield shifts in comparison with those of hepta-acetyltiliroside

Table 1. ^{1}H-NMR spectra of compounds **1a**, **2** and **2a**.

H	**1a**	**2**	**2a**
6	6.77 d (2)	6.20 d (2)	6.59 d (2)
8	7.21 d (2)	6.42 d (2)	6.79 d (2)
2′,6′	7.96 d (8)	7.98 d (8)	7.96 d (8)
3′,5′	7.11 d (8)	6.75 d (8)	7.11 d (8)
1″	5.53 d (8)	5.20 d (8)	5.53 d (8)
2″	5.17 dd (9, 8)	3.50 m	5.17 dd (9, 8)
3″	5.29 t (9)	3.50 m	5.29 t (9)
4″	5.07 t (9)	3.50 m	5.07 t (9)
5″	3.67 m	3.50 m	3.67 m
6″	4.08 m	4.30 dd (11.9 and 1.9)	4.08 m
		4.15 dd (11.9 and 6.8)	
2‴,6‴	7.50 d (8)	7.22 d (8.5)	7.50 d (8)
3‴,5‴	7.16 d (8)	6.71 d (8.5)	7.16 d (8)
7‴	7.51 d (16)	7.35 d (15.9)	7.51 d (16)
8‴	6.24 d (16)	6.03 d (15.9)	6.24 d (16)
O$\underline{C}H_3$	–	3.80 s	3.80 s
$\underline{C}OCH_3$	2.46–2.02	–	2.46–2.02

Recorded in $CDCl_3$ (**2** was recorded in DMSO-d_6), J (Hz) in parentheses.

(**1a**) [1] (table 1). The C-H HETCOR spectrum of **1** and **2** showed couplings between H-6 and C-6, H-8 and C-8, as well as H-2′,6′ and C-2′,6′, and finally between H-3′,5′ and C-3′,5′ which confirmed unambiguously the chemical shifts of the H-6, H-8, H-2′,6′ and H-3′,5′ [3, 31]. All the above data allowed us to confirm that compound **2** has never been reported previously and that it is a 7-methylether tiliroside.

Table 2. ^{13}C-NMR spectra of compounds **1a**, **2** and **2a**.

C	**1a**	**2**	**2a**
2	156.7	156.6	157.1
3	137.1	133.1	137.2
4	172.1	177.5	172.3
5	151.1	164.9	151.2
6	113.5	97.8	108.5
7	158.5	164.9	158.5
8	108.9	92.1	98.6
9	156.2	159.7	156.2
10	115.8	104.7	115.8
1′	128.2	120.6	128.1
2′,6′	131.8	130.8	130.3
3′,5′	122.1	115.1	122.1
4′	152.3	160.1	152.3
1″	99.6	100.6	99.6
2″	73.4	74.1	73.4
3″	73.5	76.2	73.5
4″	68.5	70.1	65.8
5″	72.7	74.1	72.7
6″	61.6	62.9	61.6
1‴	132.9	124.8	132.8
2‴,6‴	129.3, 129.4	130.1	129.3, 129.4
3‴,5‴	121.4, 121.5	115.6	121.4, 121.5
4‴	153.6	159.0	153.4
7‴	144.1	144.4	144.1
8‴	117.5	113.4	117.5
9‴	165.9	166.0	165.6
O$\underline{C}H_3$	–	55.9	56.1
$\underline{C}OCH_3$	169.1–169.4	–	168.9–169.4
CO$\underline{C}H_3$	20.6–21.1	–	20.6–21.2

Recorded in $CDCl_3$ (**2** was recorded in DMSO-d_6).

7-Methylether tiliroside (**2**). Yellow powder; $[\alpha]_D^{25} + 67.8$ (c. 0.12, MeOH). UV (MeOH) nm: 268, 315; ($AlCl_3$) 275, 306, 396; ($AlCl_3$ + HCl) 275, 304, 393; (MeONa) 267, 367; (NaOAc) 268, 315; (NaOAc + H_3BO_3) 268, 315. ^{1}H-NMR see table 1. ^{13}C-NMR see table 2. Positive ion FAB-MS m/z $[M + 1]^+$, 609.

Biotransformation of product 1 to product 2. Very small amounts of substance **1** were taken up by *A. nidulans* cells until the early exponential phase of the culture, as most of the substance (>95%) remained unchanged in the supernatant. It was well after the mid-exponential phase that the amounts of uptaken **1** increased to 25 to 30% and could be detected in the cytosolic content. This increase in the uptake of **1** continued until the beginning of the stationary phase (65%) and 10 h later, cultures reached maximum uptake values, i.e. 92%. Until late exponential phase, the amounts of **1** found in the cytosolic contents of *A. nidulans* mycelial cells and those remaining in the supernatant were in almost direct correlation. This possibly indicates the absence or low activity of an extracellular enzyme capable of degrading **1** in the supernatant. In the beginning of the stationary phase the first traces of substance **2** were detected in cells; and this, together with the fact that intracellular and extracellular amounts of **1** did not add up to the initial amounts provided, was considered as strong evidence that *A. nidulans* cells had started the biodegradation and biotransformation of uptaken **1**. Similarly, the time of maximum uptake of **1** coincided with the maximal amounts of substance **2** detected by TLC, and confirmed by UV-Vis quantitative determination. Under the same conditions, small traces of other substances were also observed, but these were never found in sufficient amounts to determine their nature. When the above optimum conditions were applied to large scale experiments, about 40% of substance **1** was found to remain in an unchanged form. The remaining 60% of substance **1** had therefore obviously been converted to other products. Amongst these, the most prominent was substance **2**, but at only 5% of the total **1** taken up in the cytosolic content. It should also be noted that substance **2** was never detected, even in traces, in the supernatant at any stage of growth and it is concluded that this substance is not secreted into the medium and that it remains in the cells. Thus, all the above data confirm that *A. nidulans* metabolized tiliroside (**1**) to 7-methyl tiliroside (**2**).

Cytotoxic activity and effect on DNA synthesis. Flavonoids are one of the major classes of natural products, with widespread distribution and a broad spectrum of biological activities [4]. They have been tested against cancer cell lines, showing promising effects in many cases [32, 33]. We examined the cytotoxic activity of compounds **2** and **2a** against fourteen human leukemic cell lines. It was found that compound **2** was inactive in all cell lines tested, at doses as high as 50

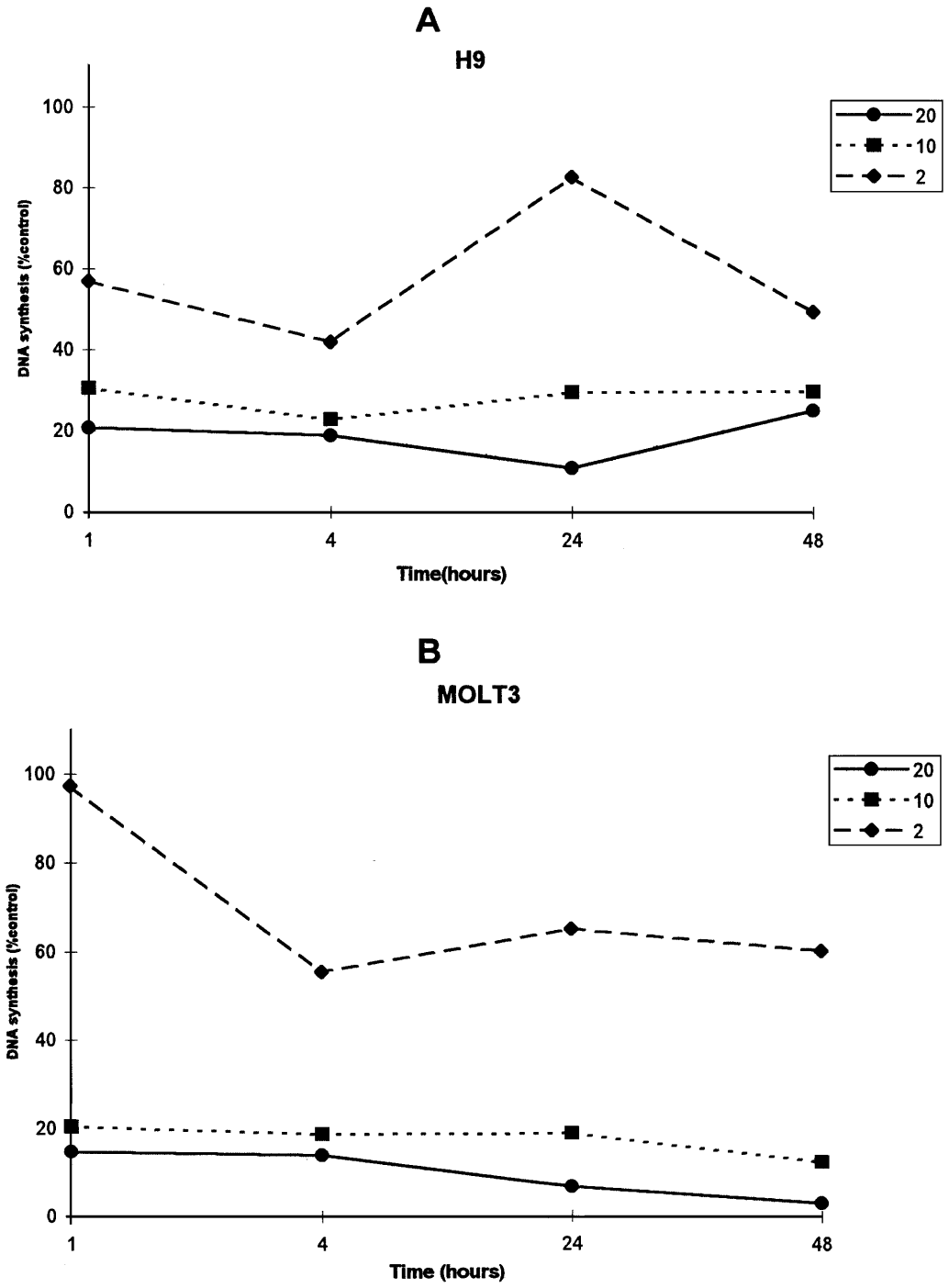

Figure 1. Effect of compound **2a** on DNA synthesis in T cell lines, H9 (lymphoblast, CD3 + CD4+) (*A*) and MOLT3 (ALL, CD4 + CD8+) (*B*). Various concentrations of compound **2a** were added and DNA synthesis was assayed as described after 1, 4, 24, 48 h. The values represent means of three independent experiments run in triplicate. SD never exceeded 15% of the mean value.

μg/ml, and for time periods as long as 48 h. It was therefore considered unnecessary to examine further the effect of this compound on DNA synthesis. On the contrary, its acetylated product (compound **2a**) exhibited a better cytotoxic activity. Its major cytotoxic effect was observed on the promyelocytic HL60 cell line with an LD_{50} below 20 μg/ml (18.5 μg/ml), followed by DAUDI (25.1 μg/ml), HUT78 (40 μg/ml) and MOLT3 (45.8 μg/ml) cell lines. The remaining 10 leukemic cell lines seem to be resistant to the influence of compound **2a**, at doses as high as 50 μg/ml.

Compound **2a** was tested further for a possible antiproliferative activity. In order to have a nonsensitive cell line as negative control, we used both sensitive and nonsensitive cell lines of the B and T lineage. In the four cell lines examined (DAUDI and JIYOYE from the B

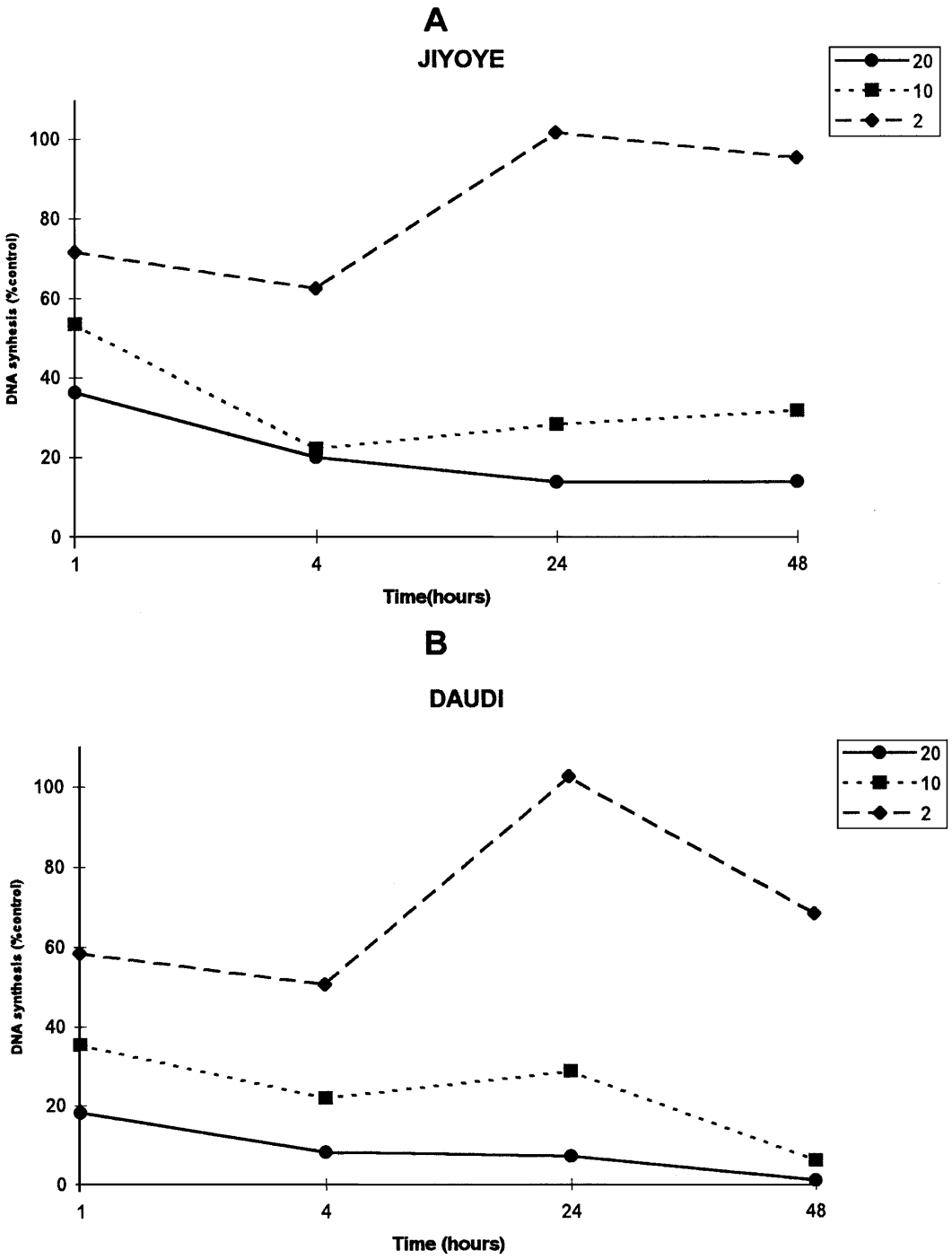

Figure 2. Effect of compound **2a** on DNA synthesis in B cell lines, JIYOYE (Burkitt lymphoma with herpes-like virus) (*A*) and DAUDI (Burkitt lymphoma with Epstein-Barr virus) (*B*). As in T cell lines various concentrations of compound **2a** were added and DNA synthesis was assayed after 1, 4, 24, 48 h. The values represent means of three independent experiments run in triplicate. SD never exceeded 17% of the mean value.

and MOLT3 and H9 from the T lineage), **2a** exhibited a significant antiproliferative activity. This flavonoid suppressed DNA synthesis at doses lower than 20 μg/ml, even in those cells where its cytotoxic activity was estimated to be at concentrations greater than 50 μg/ml. The inhibition effect began as early as 1 h after the addition of **2a**. DNA synthesis declined more at 20 μg/ml or remained at very low levels at 10 μg/ml until the end of the incubation period. At 2 μg/ml suppression of DNA synthesis was observed 1 h after the addition of compound **2a** in DAUDI, JIYOYE and H9 and after 4 h of incubation in MOLT3. In two cell lines (DAUDI and JIYOYE), DNA synthesis recovered after 24 h of incubation to 100% of the control level, but only

in JIYOYE cells did it remain at this level; in DAUDI it fell again to a level lower than 80% of the control. The above results, summarized in figures 1 and 2, show that **2a** suppresses DNA synthesis in a dose- and time-dependent manner. This suggests that the flavo-noid **2a** may also affect the cell cycle progression and growth of cells as has been already observed with other flavonoids such as quercetin [34, 35] and genistein [36]. The mechanism of action of compound **2a** as an antiproliferative agent is still under investigation.

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