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A New Class of Semisynthetic Anthracycline Glycoside Antibiotics Incorporating a Squaric Acid Moiety

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Abstract Treatment of the squaric acid amide esters (**7**, **9**) of anthracycline glycoside antibiotics with aliphatic and aromatic primary and secondary amines, amino acids, peptides and aminodeoxy sugars furnished the new asymmetric diamides **16**∼**19**, **25**∼**30**, **32**, **34** and **38**∼**40** in stereoselective reactions which do not require protecting group-manipulations. The IC_{50}=0.12 μM value measured for daunorubicin (**1**) on human leukemia (HL-60) cells is comparable to those obtained for the daunomycin-L-leucyl squaric acid diamide (**30**, IC_{50}=0.18 μM) and the corresponding D-galactosamine derivative (**40**, IC_{50}=0.22 μM).

Keywords anthracycline glycoside antibiotics, squaric acid amides, HL-60 activity

Introduction

The anthracycline glycoside antibiotics (**1**∼**3**) are extremely significant and useful agents in antitumor chemotherapy. To reduce or avoid their cardiotoxic side-effect many semisynthetic derivatives have been prepared, and the most important representatives are those modified at the aminodeoxy sugar moiety (L-daunosamine). Extensive studies have been carried out to determine the influence of the configuration of the aminodeoxy sugar unit (L-*lyxo*>L-*arabino*>L-*ribo*>L-*xylo*) [1∼3], its substituents [4∼8] and ring-size [9] on the DNA-affinity, antitumor activity and cardiotoxicity of the new (stereo)isomers.

By the acylation [10], alkylation [11], and formation of an enamino analogue [12] at the C-3′ primary amino group, as well as incorporation of this latter moiety into various heterocyclic rings (morpholine [13, 14], oxazoline [15], triazole [16], *etc.*) a huge number of semisynthetic antibiotics have been synthesized. Replacement of the α-L-daunosaminyl unit with L-acosamine (with L-*arabino* configuration) epirubicin (**4**) was obtained, which was introduced into chemotherapy. Of the amino-acyl and heterocyclic analogues, *N*-L-leucyl-adriamycin (**5**) [10] and 3′-deamino-3′-morpholinyl-pirarubicin (**6**) [11], respectively, possess the most significant antitumor effect (Fig. 1).

In a recent work, Sztaricskai and coworkers have successfully applied squaric acid esters, introduced to organic chemistry by Tietze *et al.* [17], for the chemical synthesis [18] of the squaric acid amide esters (**7**∼**9**) and the corresponding covalent dimers (**10**∼**13**) of the anthracycline glycoside antibiotics **1**∼**3** (Fig. 2).

In this paper an extension of our previous work is described, which is aimed at the synthesis of new, asymmetric diamide derivatives, and investigation of their effect on human leukemia (HL-60) cells.

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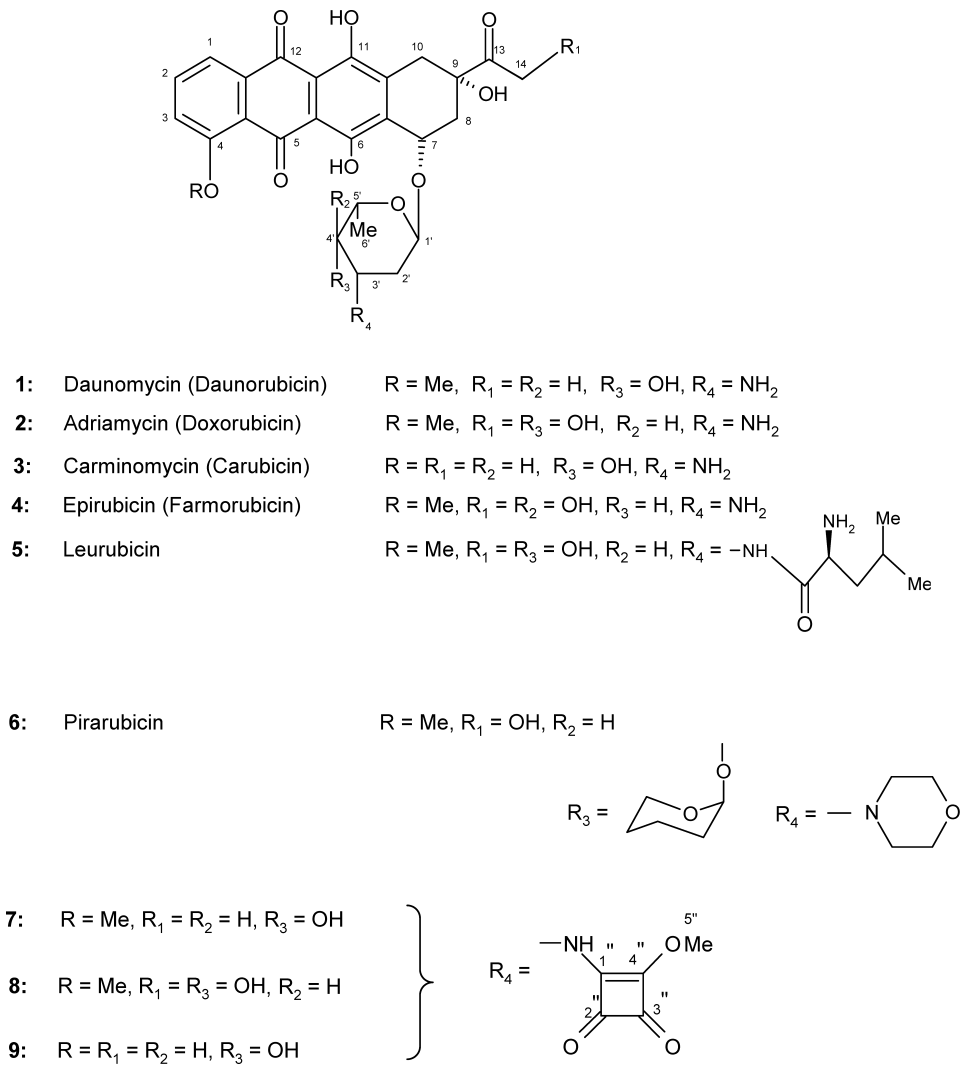
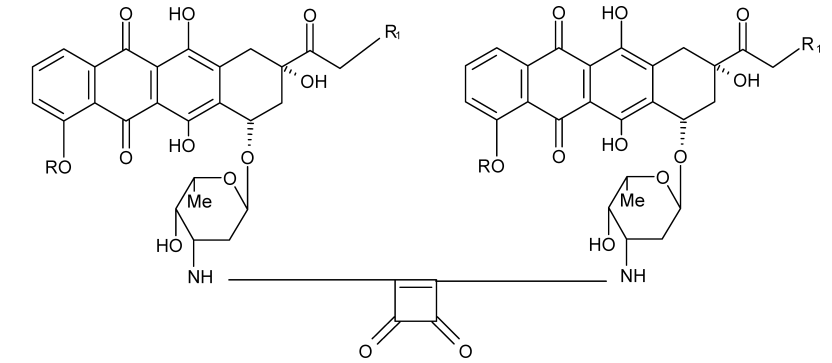


Fig. 1 Important representatives of the anthracycline glycoside antibiotics.

Results and Discussion

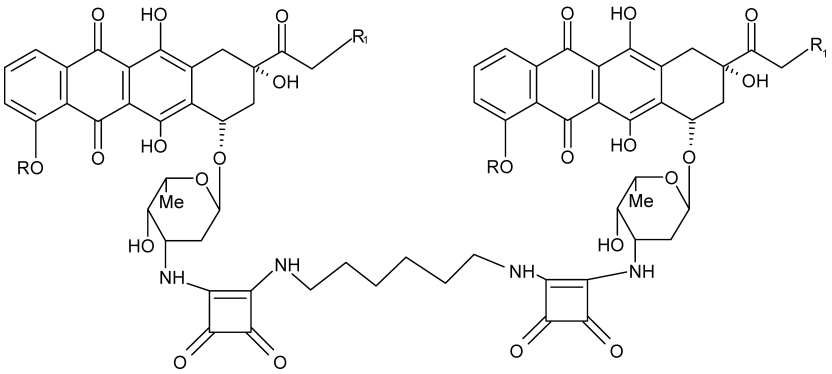
The reaction of the squaric acid amide esters (**7** and **9**) of daunomycin and carminomycin, respectively, with 4-phenylbenzylamine (**14**) was rather sluggish, whereas with 6-amino-1-hexanol (**15**) it was much faster to furnish the asymmetric diamides **16**∼**19** with good yields in each case (Scheme 1). Introduction of the aromatic or aliphatic side-chains greatly enhanced the lipoid-solubility of these semisynthetic antibiotic molecules. The physico-chemical properties and ^{1}H- and ^{13}C-NMR data of the synthesized new compounds are presented in Tables 1, 2 and 3, respectively.

The advantageous pharmacological properties of the *N*-leucyl derivative (**5**, Leurubicin® [10]) of adriamycin, introduced earlier, prompted us to perform modification of daunomycin also with this amino acid involving squaric acid. Thus, direct condensation of the squaric acid amide ester **7** with the amino acids **20** and **24**, and with the peptides **21**∼**23**, and also the similar ester **9** with triglycine gave the new semisynthetic antibiotics **25**∼**30** (Scheme 1). The pure products could be isolated with excellent yields by means of column chromatography. The desired amides with triglycine formed with practically the same yield from daunomycin and carminomycin, indicating that the presence of the free phenolic hydroxyl group, or its substitution with a methyl group do not influence the reaction. Tables 1 and 4 contain the molar masses determined with MALDI-TOF and the ^{1}H- and ^{13}C-NMR



10 : R = Me, R_1 = H

11: R = Me, R_1 = OH



12: R = Me, R_1 = H

13: R = Me, R_1 = OH

Fig. 2 Covalent dimers of the anthracycline glycoside antibiotics.

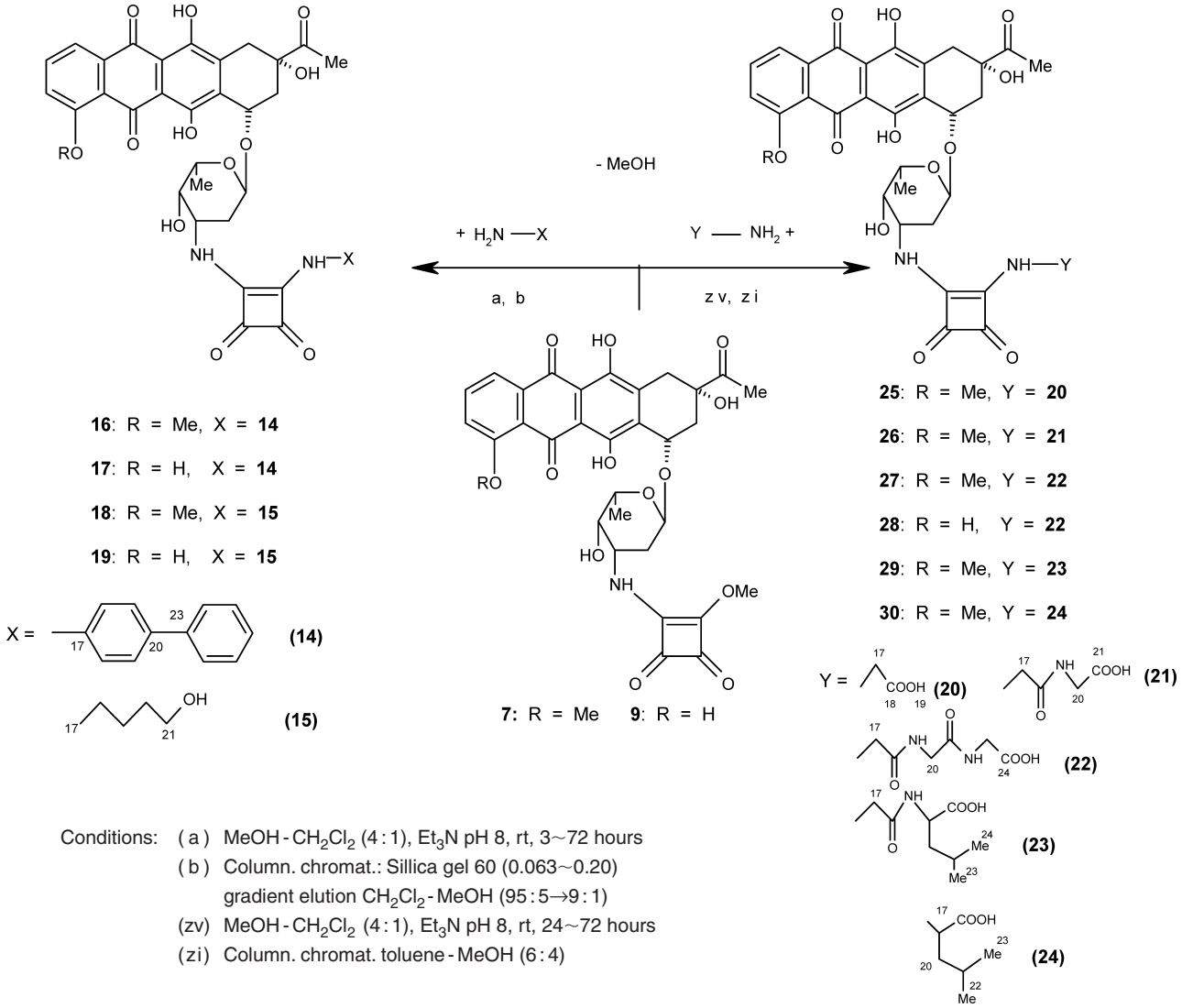
data, respectively, which substantiate the structures of the products.

Reports in the literature indicate that the *N*-morpholinyl derivatives of the anthracycline glycosides (**6**, pirarubicin) [11] are more efficient antitumor agents than the parent antibiotics, and the piperazine-compounds are essential building components of numerous drug-molecules. These encouraged us to prepare the asymmetric diamides **32** and **34** (Scheme 2) by treatment of the daunomycin squaric acid amide ester (**7**) with morpholine (**31**) and 1-benzylpiperazine (**33**). Although the reaction times with these secondary amines were longer, the desired semisynthetic antibiotics could be obtained with 77～79% yield (see Tables 1 and 5).

It is well-known that the various squaric acid derivatives possess low solubility in water [17]. However, introduction of carbohydrate moieties would enhance the water-solubility and decrease the disadvantageous toxic effects.

The reaction of **7** with D-glucosylamine (**35**) and D-glucosamine (**36**) at room temperature for 5 days resulted in **38** and **39**, and the same reaction with D-galactosamine (**37**), to give **40**, required 10 days (Scheme 2). Following column chromatographic purification, **38** and **39** were obtained with low and moderate yield, respectively, but the yield for **40** was excellent. The physico-chemical properties and the ^{1}H- and ^{13}C-NMR data for the antibiotic analogues **38**～**40** are collected in Tables 1 and 6, respectively.

As expected, the solubility of the products in water was enhanced: while 1.0 mg of the squaric acid amide ester of daunomycin (**7**) dissolved only in a 1 : 1 (v/v) DMSO-water



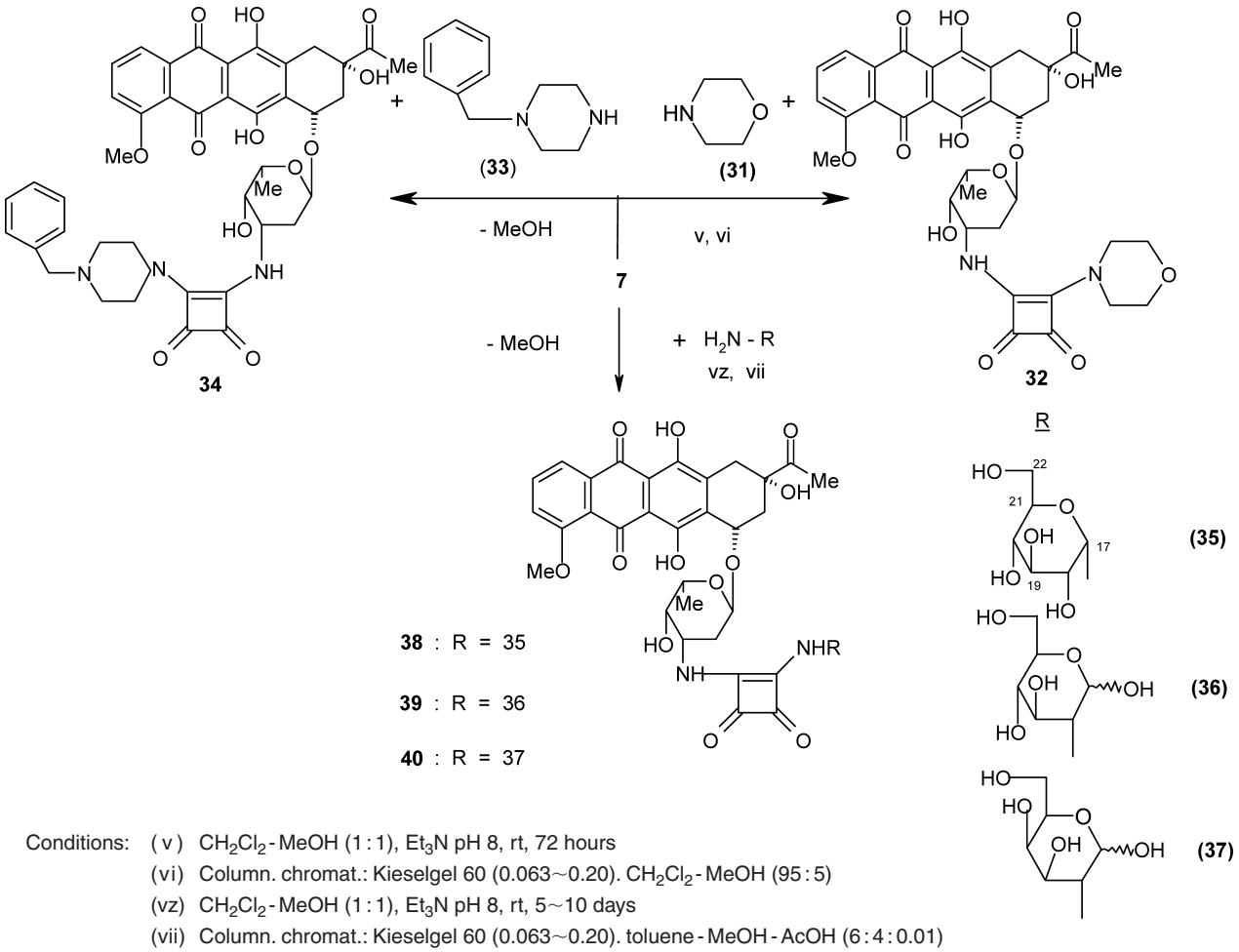
Scheme 1 Reactions of squaric acid amide esters of anthracycline antibiotics with primary amines, amino acids and peptides.

mixture (0.5 ml), the same amount of **38**~**40** was soluble in 0.5 ml of water.

In Vitro Antitumour Effect and Discussion

The results of the *in vitro* antitumor effect of the prepared new semisynthetic antibiotics on human leukemia 60 (HL-60) cells are summarized in Table 7. Comparison of the IC_{50} values obtained for the asymmetric diamides derived from daunomycin and carminomycin with 4-phenyl-benzylamine and 6-amino-1-hexanol shows that the carminomycin derivatives **17** and **19** are active in a concentration of one order of magnitude lower than the corresponding daunomycin analogues (**16** and **18**). The most active molecule is the daunomycin-L-leucyl-squaric diamide (**30**) possessing an IC_{50} value of 0.18 μM, which is very close to that (0.12 μM) measured for daunomycin (daunorubicin) introduced to clinical cancer chemotherapy. When an L-leucylglycine dipeptide unit was attached to daunomycin through a squaric acid molecule, the activity of the substance (**29**) decreased by more than one order of magnitude, and very similar results were observed for the daunomycin-diglycyl squaric acid diamide **26**.

The difference between the biological activities of the squaric acid diamides **27** and **28** of daunomycin and carminomycin, incorporating the triglycine unit, is rather surprising: the latter derivative is more effective. Biological studies [18] of the previously prepared covalent dimers, derived from carminomycin and another anthracycline



Scheme 2 Reactions of squaric acid amide esters of anthracycline antibiotics with secondary amines and aminodeoxy sugars.

glycoside antibiotics, gave similar results. At the same time, the activity of the daunomycin-morpholinyl squaric acid diamide (**32**) is one order of magnitude lower than that of the *N*-benzylpiperazinyl derivative **34**. Of the substances synthesized by the application of aminodeoxy sugars, the IC_{50} value (0.22 μM) measured for the daunomycin-D-galactosamine squaric acid diamide (**40**) is the most outstanding. Compared to this compound (**40**), **38** and **39** were found active on HL-60 cells only in three times higher concentration, and this difference can only be explained by the opposite configuration at carbon C-4 of D-glucosamine (**36**) and D-galactosamine (**37**).

Experimental

The solutions were evaporated under diminished pressure at 35~37°C on a Büchi R-114 rotary evaporator. For monitoring of the reactions, and homogenity check TLC and HPLC were used. Related, and the UV spectral and MALDI-TOF data are presented in Table 1. For NMR spectroscopy, a Bruker DRX-500 instrument operating at 500.13 MHz and 125.79 MHz frequencies, respectively, for the ^{1}H and ^{13}C nuclei was used (see Tables 2~6). Internal TMS was the reference material. Tipically, ^{1}H, ^{13}C, and 2D COSY and HSQC spectra were recorded for assignment, sometimes augmented with TOCSY and HBMC spectra.

Table 1 Physico-chemical properties of the squaric acid asymmetric diamides of the anthracycline antibiotics

Asymmetric diamides of squaric acid	Yied (%)	HPLC R_T	TLC Rf	Formula of compounds	Molecular weight		UV λ_{max} nm (DMSO)
					Calculated	Measured MALDI−TOF $(M+Na)^+$	
16	61.8	22.87	(1) 0.40	$C_{44}H_{40}N_2O_{12}$	788.78	811.46	295
17	65.8	24.10	(1) 0.17	$C_{43}H_{38}N_2O_{12}$	774.45	797.42	258
18	73.0	17.22	(1) 0.40	$C_{37}H_{42}N_2O_{13}$	722.73	745.47	294
19	69.8	18.39	(1) 0.20	$C_{36}H_{40}N_2O_{17}$	708.70	731.43	296
25	95.8	16.07	(2) 0.15	$C_{33}H_{32}N_2O_{14}$	680.61	703.16	295
26	93.0	15.48	(2) 0.12	$C_{35}H_{35}N_3O_{15}$	737.65	760.00	252
27	97.1	15.04	(2) 0.32	$C_{37}H_{38}N_4O_{16}$	794.70	817.64	294
28	97.6	16.04	(2) 0.35	$C_{36}H_{36}N_4O_{16}$	780.68	803.37	294
29	92.0	17.57	(2) 0.36	$C_{39}H_{43}N_3O_{15}$	793.75	816.21	297
30	68.1	19.33	(2) 0.27	$C_{37}H_{40}N_2O_{14}$	736.71	759.00	297
32	78.9	—	(3) 0.44	$C_{36}H_{36}N_2O_{13}$	692.66	715.48	291
34	76.7	—	(3) 0.42	$C_{42}H_{43}N_3O_{12}$	781.78	804.42	293
38	29.1	14.59	(2) 0.45	$C_{37}H_{40}N_2O_{17}$	784.71	807.41	293
39	89.0	14.36	(2) 0.20	$C_{37}H_{40}N_2O_{17}$	784.71	807.39	252
40	49.1	14.42	(2) 0.26	$C_{37}H_{40}N_2O_{17}$	784.71	807.02	288

TLC: Silica gel 60 F_{254} (Merck); Solvent systems: (1) CH_2Cl_2 - MeOH (9 : 1); (2) toluene - MeOH - AcOH (6 : 4 : 0.01); (3) CH_2Cl_2 - MeOH (95 : 5).
HPLC: instrument: Waters 600, UV detection 230 nm; column: Lichrospher RP-8 (4×250 mm/mm; 10 μm); Injection volume: 20 μl (0.1 mg/ml); Eluents: A: CF_3COOH - H_2O (pH 2.65), B: MeCN gradient elution; 0 minute 10% B→30 minutes 90% B.
MALDI-TOF mass spectra: Bruker Biflex III instrument (the samples were dissolved in DMSO with a 2,5-dihydroxybenzoic acid matrix, concentration: 20 mg/ml, concentration of the components: 5 μg/ml, nitrogen laser). Detection of the positive ions was made in the reflecton mode.
The UV spectra were recorded with a Perkin-Elmer λ 11 instrument (±2 nm, absorbance accuracy: 0.001 Å).

(For some compounds direct ^{13}C-NMR spectra were difficult to acquire because of poor solubilty.) Assignments were corroborated with chemical shift prediction of the ACD program [19] and according to our previous work [18].

General Method for the Synthesis of Asymmetric Diamides of Squaric Acid

The anthracycline antibiotic squaric acid amide esters **7** and **9** (0.10∼0.15 mmol) were taken up in a 4 : 1 dichloro-methane - methanol mixture at room temperature, and if necessary, 1∼2 drops of *N*,*N*-dimethylformamide were added for complete dissolution.The apparent pH of the solution was adjusted to *ca.* 8 by addition of a few drops of triethylamine and then 0.10∼0.15 mmol of the reaction partner carrying a primary or secondary amino group was added. The reaction mixtures were monitored by means of TLC. After completion of the transformation, the mixtures were applied onto the surface of a small amount of Silica gel 60 by evaporation, and chromatographed on Silica gel 60 columns wich were eluted with the eluent systems indicated in Schemes 1 and 2.

The physical characteristics (Rf and R_T values), and the UV and mass spectral data are shown in Table 1, and the ^{1}H- and ^{13}C-NMR data are collected in Tables 2∼6.

In Vitro Antitumour Effect of Daunomycin and Carminomycin Derivatives

The HL-60 human leukemia cell line was cultured at 37°C under 5% CO_2 in RPMI-1640 medium containing 10% FBS and 2 mM glutamine.

For cytotoxicity studies cells were placed in a 96-well plate with each well containing 5×10^3 cells. After incubation at 37°C for 24 hours, the cultured cells were treated with the daunomycin or carminomycin derivatives dissolved in DMSO diluted with serum-free RPMI-1640 medium (1 : 39, v/v) for 3 hours. The compounds were used at the $c=5\times10^{-4}$ to 1×10^{-9} M range. In control experiments the cells were treated with DMSO diluted with serum - free medium (1 : 39, v/v) at 37°C for 3 hours. After incubation, the cells were washed with serum - free medium three times and serum-containing medium was added to the

Table 2 ^{1}H- and ^{13}C-NMR spectral data of the squaric acid amide esters of anthracycline antibiotics

Atom number	Group	Compounds					
		7 ($CDCl_3$)		**8** (DMSO+$CDCl_3$)		**9** ($CDCl_3$+DMSO=10 : 1)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
1	CH	8.04	120.23	7.92	120.06	7.75	119.92
2	CH	7.80	136.2	7.742	136.4	7.65	137.51
3	CH	7.41	118.9	7.392	119.4	7.21	125.17
4	C	—	161.5	—	161.48	—	162.8
4a	C	—	121.8	—	121.06	—	116.31
5	C	—	187.46	—	187.33	—	190.78
5a	C	—	(111.82)[c]	—	(111.64)[c]	—	(110.67)[c]
6	C	—	(156.16)[b]	—	(165.65)[b]	—	(157.12)[b]
6a	C	—	(134.39)[a]	—	(134.78)[a]	—	(134.75)[a]
6b	OH	(13.29)	—	13.2	—	12.81	—
7	CH	5.40	68.68	5.23	68.54	5.20	68.76
8	CH_2	2.34/2.18	35.33	2.26/2.09	36.25	2.25/2.04	35.69
9	C	—	77.07	—	76.85	—	76.61
10	CH_2	3.27/2.98	34.18	3.11/2.98	34.25	3.09/2.95	33.91
10a	C	—	(134.94)[a]	—	(134.54)[a]	—	(133.65)[a]
11	C	—	(156.62)[b]	—	(155.91)[b]	—	(157.12)[b]
11a	C	—	(111.95)[c]	—	(111.76)[c]	—	(111.51)[c]
11b	OH	14.05	—	13.97	—	13.36	—
12	C	—	187.14	—	187.11	—	186.44
12a	C	—	135.88	—	135.66	—	137.44
13	C	—	212.02	—	213.98	—	212.07
14	CH_3, CH_2	2.45	(CH_3) 25.18	4.684	(CH_2) 65.45	2.34	(CH_3) 25.07
15	CH_3	4.10	57.1	3.95	57.15	—	—
1′	CH	5.61	99.45	5.44	99.83	5.42	100.5
2′	CH_2	2.04/1.86	31.33	2.05/1.67	30.07	2.07/1.72	30.81
3′	CH	4.23	51.06	3.88	51.62	3.88	51.66
4′	CH	3.78	70.09	3.55	70.05	3.58	69.55
5′	CH	4.17	67.85	3.99	68.22	4.05	68.2
6′	CH_3	1.37	17.08	1.22	17.45	1.24	17.38
7′	OH	3.51	—	—	—	—	—
8′	NH	6.82	—	—	—	—	—
1″	C	—	170.22	—	172.33	—	172.31
2″	C	—	190.03	—	189.46	—	189.46
3″	C	—	182.99	—	183.63	—	183.66
4″	C	—	178.46	—	177.56	—	177.57
5″	CH_3	4.33	61.14	4.16	60.64	4.18	60.66

Assignments in parenthesis are exchangeable.

cells. After 4 days at 37°C, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay [20, 21] was carried out. The yellow solution of MTT at a concentration of 2 mg/ml was added to each well. The purple crystal obtained was dissolved in DMSO and the optical density (OD) of the samples was measured at $\lambda = 540$ nm using ELISA Reader (Labsystems MS Reader, Finland). The percent of antitumour effect was calculated using the following equation:

$$\text{Antitumour effect } \% = \left(1 - \text{OD}_{\text{treated}} / \text{OD}_{\text{control}}\right) \times 100,$$

where $\text{OD}_{\text{treated}}$ and $\text{OD}_{\text{control}}$ correspond to the optical

Table 3 ^{1}H- and ^{13}C-NMR spectral data of the asymmetric diamides of squaric acid

Atom number	Group	Compounds							
		16 (DMSO) (300 K)		**17** (DMSO) (300 K)		**18** (DMSO) (327 K)		**19** (DMSO) (327 K)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
29	NH	7.90	—	7.90	—	7.42	—	7.47	—
16	CH_2	4.73	47.34	4.74	47.34	3.48	44.10	3.49	44.12
17	C/CH_2	—	138.90	—	140.22	1.511	31.54	1.512	31.56
18	CH/CH_2	7.39	129.05	—	—	1.30	26.65	1.31	25.97
19	CH/CH_2	7.65	127.85	7.65	127.84	1.31	25.97	1.31	26.65
20	C/CH_2	—	140.23	—	140.60	1.42	33.27	1.42	33.28
21	CH/CH_2	7.65	127.85	7.65	127.84	3.39	61.54	3.38	61.55
22	CH/OH	7.39	129.05	—	—	—	—	—	—
23	C	—	140.61	—	—	—	—	—	—
24	CH	7.63	127.48	7.63	127.47	—	—	—	—
25	CH	7.45	129.79	—	129.78	—	—	—	—
26	CH	7.35	128.33	7.35	128.33	—	—	—	—
27	CH	7.45	129.79	—	129.78	—	—	—	—
28	CH	7.63	127.48	7.63	127.47	—	—	—	—

Due to the small changes, in the following no data for the skeletone are given.

Table 4-1 ^{1}H- and ^{13}C-NMR spectral data of the asymmetric diamides of squaric acid

Atom number	Group	Compounds			
		25 (MeOD) (298 K)		**26** (MeOD) (300 K)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
17	CH_2	4.77	48.44	4.21	46.92
20	CH_2	—	—	3.562	44.35

Table 4-2 ^{1}H- and ^{13}C-NMR spectral data of the asymmetric diamides of squaric acid

Atom number	Group	Compounds			
		27 (DMSO) (300 K)		**28** (DMSO) (300 K)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
16	NH	7.60	—	7.61	—
17	CH_2	3.45	44.47	3.46	44.35
18	C	—	173.15	—	178.87
19	NH	8.50	—	8.45	—
20	CH_2	4.22/4.32	46.85	4.23/4.28	46.80
21	C	—	171.81	—	173.18
22	NH	8.69	—	8.65	—
23	CH_2	3.81	43.28	3.78	43.24
24	C	—	175.08	—	174.36

Table 4-3 ^{1}H- and ^{13}C-NMR spectral data of the asymmetric diamides of squaric acid

Atom number	Group	Compounds			
		29 (MeOD) (300 K)		**30** (MeOD) (300 K)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
17	CH_2/CH	5.49	56.77	5.24	58.63
20	CH/CH_2	4.37	44.59	2.26/2.40	45.07
21	CH_2/CH	n.a.	n.a.	2.39	25.89
22	CH/CH_3	2.40	25.38	1.63	24.13
23	CH_3	1.67	23.91	1.64	24.13
24	CH_3	1.65	23.39	—	—

Table 5 ^{1}H- and ^{13}C-NMR spectral data of the asymmetric diamides of squaric acid

Atom number	Group	Compounds			
		32 (MeOD) (300 K)		**34** ($CDCl_3$+DMSO) (300 K)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
17	CH_2	3.74	47.54	n.a.	n.a.
18	CH_2	3.73	66.84	2.62	52.83
20	CH_2	3.73	66.84	2.62	52.83
21	CH_2	3.74	47.54	n.a.	n.a.
22	CH_2	—	—	3.64	62.72
24	CH	—	—	7.35	129.81
25	CH	—	—	7.31	128.79
27	CH	—	—	7.31	128.79
28	CH	—	—	7.35	129.81

Due to deuteration in MeOD, the OH and NH signals are not detectable (n.a.).

Table 6 ^{1}H- and ^{13}C-NMR spectral data of the asymmetric diamides of squaric acid

Atom number	Group	Compounds					
		38 (DMSO+MeOD)		**39** (DMSO+MeOD)		**40** (DMSO+MeOD)	
		^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
17	CH	4.87	88.37	5.05	92.50	5.23	92.02
18	CH	3.72	64.31	3.86	59.01	4.25	55.21
19	CH	3.19	79.06	3.52	72.28	3.84	68.93
20	CH	3.22	78.08	3.64	72.81	3.96	70.14
21	CH	3.13	70.02	3.19	71.49	3.65	63.09
22	CH_2	3.45/3.62	61.91	3.55/3.62	61.91	3.74	62.10

Table 7 The effect of the new anthracycline derivatives on HL-60 cells *in vitro*

Compound number	IC_{50}* (μM)
Daunomycin · HCl (**1**)	0.12
16	19.96
17	1.57
18	15.65
19	1.31
25	2.54
26	5.70
27	19.60
28	2.69
29	5.75
30	0.18
32	29.21
34	3.39
38	0.58
39	0.65
40	0.22

* IC_{50} values were determined as described in the Experimental section and were calculated as an average of three measurements.

densities of the treated cells and the control cells, respectively, at $\lambda = 540$ nm. The results obtained from *in vitro* cytotoxicity measurements were analysed using sigmoidal curve fitting.

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