Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4783–4789
Bioorganic & Medicinal Chemistry Letters

Formation of squaric acid amides of anthracycline antibiotics. Synthesis and cytotoxic properties

Anna Tevyashova,[a,†] Ferenc Sztaricskai,[a,*] Gyula Batta,[a] Pál Herczegh[a] and András Jeney[b]

[a]*Research Group for Antibiotics of the Hungarian Academy of Sciences, and Department of Pharmaceutical Chemistry, University of Debrecen, Debrecen, PO Box 70, H-4010 Hungary*
[b]*Semmelweis University of Medicine, I. Institute of Pathology and Experimental Cancer Research, Budapest, Üllői út 26, H-1085 Hungary*

Received 10 February 2004; revised 25 May 2004; accepted 16 June 2004
Available online 20 July 2004

Abstract—The reaction of the anthracycline glycoside antibiotics **1**–**3** with the squaric acid ester **4** gave the squaric acid amide esters **5**–**7** under neutral conditions, whereas over pH 7 the products are the symmetric diamides (**8**, **9**, **11**, and **12**). Of the prepared compounds **11** was the most active on MCF-7 human mammary adenocarcinoma cells.

The anthracycline glycoside-type antibiotics[1] daunomycin (**1**; Daunorubicin), adriamycin (**2a**; Doxorubicin), epirubicin (**2b**, Farmorubicin) and carminomycin (**3**; Carubicin) are widely used in cancer chemotherapy. An important drawback of the application of these antibiotics is that their antitumor activity is associated with a significant, irreversible cardiotoxic effect. The aim of the chemical modification of the anthracycline glycoside antibiotics is to synthesize less cardiotoxic analogues[2] that keep the beneficial biological action of the parent compounds. In addition, the development of new generations of antibiotics is an efficient tool in fighting against tumor resistance.

A novel, promising possibility for the synthetic modification of these antibiotics at the carbohydrate (α-L-daunosaminyl) moiety[3,4] is the reaction with squaric acid esters (**4**)[5].

Compound **4** selectively reacts with a small excess of primary or secondary amines in aqueous and/or alcoholic media and in chlorinated hydrocarbons even in the presence of alcoholic or phenolic hydroxyl groups. In neutral media, the product is always the squaric acid amide ester, whereas under more alkaline conditions (pH > 7) the corresponding symmetric diamide is produced. Thus the method of Tietze et al.[5] permits the attachment of a drug to a biomolecule through a squaric amide bond.

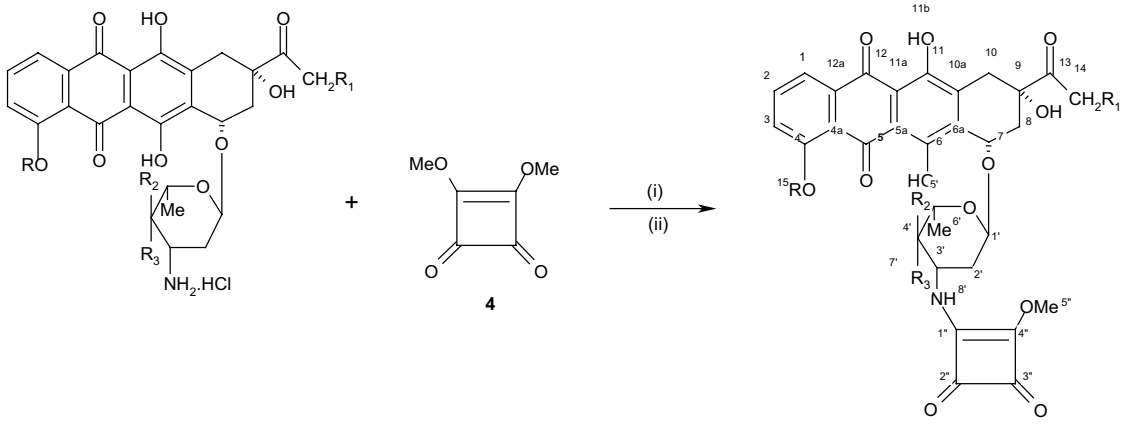
In the present paper, we report the derivatization of the natural anthracycline glycoside antibiotics with the squaric acid ester **4**, so that to make these antibiotic derivatives suitable for covalent binding to biomolecules. The biological activity of the new anthracyclines was also investigated.

Treatment of the antibiotics **1**, **2a**, and **3** with dimethyl squarate (**4**) in ethanol at room temperature for 20–42 h resulted in the squaric acid amide ester derivatives **5**–**7** (Scheme 1). It was found that the reaction of daunomycin (**1**) proceeded faster and with higher yield than that of adriamycin (**2a**) and carminomycin (**3**), which carry free primary alcoholic and phenolic hydroxyl groups at the aglycone moiety. Compounds **5**–**7** were isolated after purification by means of column chromatography (Scheme 1), and their homogeneity was checked with TLC and HPLC examinations (Table 1). The structures of the new antibiotic analogues were established by MALDI TOF (Table 1), and ^{1}H and ^{13}C NMR spectroscopic measurements (Table 2).

The 1- and 2D NMR experiments were run on a Bruker DRX 500 NMR spectrometer in solvents indicated in

* Corresponding author. Tel.: +36-52-512-900/2475; fax: +36-52-512-914; e-mail: sztarife@delfin.klte.hu
† Permanent address: Institute of New Antibiotics, Academy of Medical Sciences, B. Pirogovskaya 11, Moscow 119867, Russia.

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.06.072



1: Daunomycin; R = Me, $R_1 = R_2 = H$, $R_3 = OH$

2a: Adriamycin; R = Me, $R_2 = H$, $R_1 = R_3 = OH$

2b: Epirubicin; R = Me, $R_1 = R_2 = OH$, $R_3 = H$

3: Carminomycin; R = $R_1 = R_2 = H$, $R_3 = OH$

5: R = Me, $R_1 = R_2 = H$, $R_3 = OH$ (63 %)

6: R = Me, $R_1 = R_2 = H$, $R_3 = OH$ (51 %)

7: R = $R_1 = R_2 = H$, $R_3 = OH$ (48 %)

Scheme 1. Preparation of the squaric acid monoamides of anthracycline antibiotics. Reagents and conditions: (i) EtOH, Et_3N (pH ~ 7), 20 °C, 20–42 h; (ii) column chromatography: Kieselgel 60 (0.063–0.20 mm, Merck), $CHCl_3$–MeOH (95:5) and/or $CHCl_3$–MeOH–HCOOH (9:1:0.05).

Table 1. Characterization of the synthetic compounds by TLC, HPLC, and mass spectrometry

Number of sample	R_f[a]	R_t[b]	Molecular weight[c]	
			Calculated	Found $(M+Na)^+$
5	0.58	19.302	637.60	660.20
6	0.40	16.789	653.60	676.12
7	0.55	21.062	623.58	646.15
8	0.36	23.070	1133.09	1155.45
9	0.16	18.870	1165.09	1187.50
11	0.21	20.384	1327.27	1349.43
12	0.19	16.930	1359.24	1381.63

[a] TLC was performed on Kieselgel 60 F_{254} (Merck) with a $CHCl_3$–MeOH–HCOOH (9:1:0.05) solvent system.

[b] HPLC were performed with on a Shimadzu HPLC LC 50 instrument equipped with a Diaspher C-18 column (2.0×150 mm), UV detector: 254 mm, sample concentration: 0.05–0.2 mg/mL and injection volume 10 μL. Elutions were carried by 0.01 M H_3PO_4–MeCN, pH 2.6 at 20 °C using gradient MeCN from 10–90% (0 min—10% MeCN, 30 min—90% MeCN).

[c] MALDI-TOF MS measurements were performed with a Bruker BIFLEX III mass spectrometer. In all cases 19 KV acceleration was used. The positive ions were detected in the reflektron mode. Samples were prepared with DHB matrix dissolved in DMSO (20 mg/mL). The samples of the antibiotics were made in a concentration of 5 mg/mL. The solutions were mixed in 5:1 v/v ratio (matrix: antibiotics samples).

Table 2 and referenced to TMS observing protons at 500.13 and ^{13}C at 125.79 MHz. Spectral assignments were carried out using COSY, HSQC, TOCSY, and HMBC methods. Assignments were augmented with ^{13}C chemical shift prediction as shown for compound **5**. The general agreement between the experimental and ACD software[6] prediction is surprisingly good.

In the antibiotic molecules **5**–**7** a covalent hemi-amide bond developed between the amino group at C-3′ of daunosamine (3-amino-2,3,6-trideoxy-L-*lyxo*-hexopyranose) and dimethyl squarate **4**.

Application of compounds **5**–**7** may open new perspectives for the synthetic production of bioconjugates in which the target-recognizing unit and the anthracycline antibiotic molecule, which is responsible for the antitumor activity, are connected with a covalent bonding.

The reactive esters **5**–**7**, possessing a vinylamide structure, can be converted into covalent dimers, that is to diamides. Thus, the reaction of an excess of **1** with the monoamide ester **5** in ethanol under alkaline conditions (pH > 8) at room temperature furnished the symmetric squaric diamide (**8**) for a relatively long time (48 h). The analogous reaction of **6** and **2a** proceeded even more sluggishly, and with lower yield to afford **9** (Scheme 2), but no conversion was observed when **7** and **3** were employed. The symmetric anthracycline squaric diamides **8** and **9** were purified with column chromatography on Silicagel 60.

The presence of squaric acid in the molecules **8** and **9** is unequivocally proved by the ^{13}C chemical shift assignments to this unit, although these could be influenced by the H-bond pattern in different solutions. Also, the place of attachment at the deoxysugar (C-3′-NH_2) has a characteristic ^{13}C NMR shift.

The reaction of the daunosaminyl squaric esters **5** and **6** with the hexamethylenedianine **10** led to the antibiotic dimers **11** and **12** in which the amino groups of the two L -daunosamine units are linked with a six-carbon *n*-alkyl chain linker through covalent bondings (Scheme 3). The reactivity of **5** towards **10** at alkaline pH was similar to that observed in the case of the preparation of **8** and **9**, and column chromatographic purification

Table 2. ^{1}H and ^{13}C NMR spectra of the synthesized compounds

Atom no.	Group	Predicted ^{13}C shift ppm[d]	Compounds					
			5 ($CDCl_3$)		**6** (DMSO+$CDCl_3$)		**7** ($CDCl_3$+DMSO = 10:1)	
			^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)	^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)	^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)
1	CH	119.5	120.23	8.043	120.06	7.919	119.92	7.746
2	CH	135.3	136.21	7.803	136.41	7.7423	137.51	7.647
3	CH	118.2	118.91	7.411	119.36	7.392	125.17	7.215
4	C	160.8	161.5	—	161.48	—	162.8	—
4a	C	120.2	121.8	—	121.06	—	116.31	—
5	C	186	187.46	—	187.33	—	190.78	—
5a	C	110.5	(111.82)[c]	—	(111.64)[c]	—	(110.67)[c]	—
6	C	155	(156.16)[b]	—	(165.65)[b]	—	(157.12)[b]	—
6a	C	132.89	(134.39)[a]	—	(134.78)[a]	—	(134.75)[a]	—
6b	OH	—	—	(13.29)	—	13.20	—	12.81
7	CH	69	68.68	5.405	68.54	5.233	68.76	5.204
8	CH_2	34.8	35.33	2.34/2.18	36.25	2.266/2.092	35.69	2.252/2.045
9	C	78.8	77.07	—	76.85	—	76.61	—
10	CH_2	33.4	34.18	3.277/2.989	34.25	3.116/2.981	33.91	3.093/2.956
10a	C	133.9	(134.94)[a]	—	(134.54)[a]	—	(133.65)[a]	—
11	C	156.4	(156.62)[b]	—	(155.91)[b]	—	(157.12)[b]	—
11a	C	110.7	(111.95)[c]	—	(111.76)[c]	—	(111.51)[c]	—
11b	OH	—	—	14.05	—	13.97	—	13.36
12	C	186	187.14	—	187.11	—	186.44	—
12a	C	134	135.88	—	135.66	—	137.44	—
13	C	211.6	212.02	—	213.98	—	212.07	—
14	CH_3 (CH_2)	24.7	25.18	2.448	(CH_2) 65.45	4.684	25.07	2.336
15	CH_3	56.6	57.1	4.098	57.15	3.95	—	—
1′	CH	100.81	99.45	5.609	99.83	5.441	100.5	5.419
2′	CH_2	32.59	31.33	2.04/1.861	30.07	2.054/1.669	30.81	2.076/1.719
3′	CH	54.1	51.06	4.023	51.62	3.884	51.66	3.888
4′	CH	68.35	70.09	3.779	70.05	3.554	69.55	3.582
5′	CH	65.74	67.85	4.171	68.22	3.996	68.2	4.053
6′	CH_3	16.94	17.08	1.377	17.45	1.221	17.38	1.239
7′	OH	—	—	3.517	—	ND	—	ND
8′	NH	—	—	6.826	—	ND	—	ND
1″	C	170.71	170.22	—	172.33	—	172.31	—
2″	C	197.55	190.03	—	189.46	—	189.46	—
3″	C	195.45	182.99	—	183.63	—	183.66	—
4″	C	162.22	178.46	—	177.56	—	177.57	—
5″	CH_3	55.19	61.14	4.328	60.64	4.164	60.66	4.186

(*continued on next page*)

Table 2 (*continued*)

Atom no.	Group	Compounds							
		8 (DMSO+MeOD)		**9** (DDMSO+$CDCl_3$)		**11** ($CDCl_3$+DMSO+MeOD)		**12** ($CDCl_3$+MeOD)	
		^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)	^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)	^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)	^{13}C-chem.shift (ppm)	^{1}H-chem.shift (ppm)
1	CH	119.69	7.863	119.90	7.863	119.64	7.758	119.84	7.869
2	CH	136.61	7.801	136.80	7.801	136.32	7.706	136.06	7.363
3	CH	120.87	7.529	120.27	7.529	119.49	7.398	118.86	7.732
4	C	161.56	—	161.62	—	161.38	—	ND	—
4a	C	120.87	—	121.02	—	120.87	—	ND	—
5	C	187.27	—	187.32	—	187.01	—	ND	—
5a	C	(111.46)[c]	—	(111.53)[c]	—	(111.29)[c]	—	ND	—
6	C	(156.46)[b]	—	(156.99)[b]	—	(155.18)[b]	—	ND	—
6a	C	(135.53)[a]	—	(135.63)[a]	—	(134.91)[a]	—	(134.64)[a]	—
6b	OH	—	(13.23)	—	(13.23)	—	(13.11)	—	(13.11)
7	CH	69.79	4.992	70.68	5.018	69.91	5.044	68.96	5.227
8	CH_2	36.92	2.201/2.118	37.63	2.209/2.135	36.47	2.225/2.063	36.36	2.353/1.863
9	C	76.2	—	76.04	—	76.36	—	76.50	—
10	CH_2	32.55	2.943	33.08	2.992	32.77	2.94	33.77	3.216/3.032
10a	C	(135.04)[a]	—	(135.08)[a]	—	(135.36)[a]	—	(135.58)[a]	—
11	C	(155.01)[b]	—	(155.49)[b]	—	(156.4)[b]	—	ND	—
11a	C	(111.62)[c]	—	(111.66)[c]	—	(111.47)[c]	—	ND	—
11b	OH	—	(13.99)	—	(13.99)	—	(13.84)		(13.84)
12	C	187.25	—	187.32	—	187.01	—	187.01	—
12a	C	136.61	—	136.18	—	135.45	—	(111.29)[c]	—
13	C	212.33	—	214.43	—	212.59	—	ND	—
14	$CH_3(CH_2)$	24.41	2.249	(64.71)	(4.575)	24.51	2.287	(65.3)	(4.78)
15	CH_3	56.87	3.95	57.33	3.988	56.70	3.91	56.73	4.00
1′	CH	100.69	5.302	100.69	5.302	100.40	5.302	100.23	5.518
2′	CH_2	31.55	1.877/1.689	31.59	1.873/1.661	31.45	1.916/1.761	31.34	2.008/1.863
3′	CH	50.04	4.227	50.07	4.223	50.19	4.265	50.19	4.377
4′	CH	70.89	3.471	69.84	3.463	69.91	3.541	69.79	3.685
5′	CH	67.24	4.161	64.71	4.13	67.33	4.17	67.59	4.221
6′	CH_3	17.21	1.157	17.73	1.156	17.04	1.157	16.65	1.294
7′	OH	—	ND	—	ND	—	ND	—	ND
8′	NH	—	ND	—	ND	—	ND	—	ND
1″	C	167.72	—	167.89	—	167.48	—	ND	—
2″	C	182.77	—	183.01	—	182.88	—	ND	—
3″	C	182.77	—	183.01	—	182.88	—	ND	—
4″	C	167.72	—	167.89	—	168.51	—	168.34	—
α	CH_2	—	—	—	—	43.84	3.501	43.69	3.561
β	CH_2	—	—	—	—	30.96	1.456	30.08	1.456
γ	CH_2	—	—	—	—	25.68	1.27	24.54	1.381

ND=not detected because of low concentration.

[a,b,c]Reversible assignments.

[d] Predicted for **5**.

Table 3. Cytotoxic action of the new anthracycline compounds

Compound	Concentration (ng/mL)	Absorbance[a] MTT test	Control (%)	IC_{50} (ng/mL)
Control		1.732 + 0.201	100	
Epirubicin (Farmorubicin) **2b**	0.3	1.294 + 0.391	74.7	3.23
	3.0	0.710 + 0.214	41.0	
	30.0	0.611 + 0.161	35.3	
	300.0	0.258 + 0.127	14.9	
Carminomycin **3**	0.3	1.853 + 0.393	107.0	48.28
	3.0	1.614 + 0.241	93.2	
	30.0	1.333 + 0.258	77.0	
	300.0	0.158 + 0.028	9.1	
5	3.0	1.472 + 0.430	85.0	>300
	30.0	1.293 + 0.353	74.7	
	300.0	1.348 + 0.457	77.8	
6	3.0	1.524 + 0.153	88.0	>300
	30.0	1.619 + 0.072	93.5	
	300.0	1.604 + 0.096	92.6	
7	3.0	1.554 + 0.083	89.7	299.4
	30.0	1.403 + 0.293	81.0	
	300.0	0.801 + 0.187	46.3	
8	3.0	1.259 + 0.203	72.7	108.6
	30.0	1.052 + 0.262	60.7	
	300.0	1.727 + 0.374	42.0	
9	3.0	1.524 + 0.077	88.0	>300
	30.0	1.464 + 0.093	84.5	
	300.0	1.184 + 0.251	68.4	
11	3.0	1.801 + 0.210	104.0	87
	30.0	0.866 + 0.185	50.0	
	300.0	0.554 + 0.130	32.0	
12	3.0	1.521 + 0.062	87.0	>300
	30.0	1.492 + 0.072	86.1	
	300.0	1.427 + 0.125	82.4	

Methods: MCF-7 human mammary adenocarcinoma cells (5×10^3) were seeded in 24 multiwell plates (Greiner, Nürtingen, Germany) and grown in RPMI-1640 medium (Sigma-Aldrich Kft., Budapest) supplemented with 10% fetal calf serum plus penicillin (50 unit/mL) and streptomycin (100 μg/mL), at 37 °C in a humid atmosphere of 5% CO_2 and 95% air for 72 h. Test compounds were administered 3 h after plating the cells, after treatment for 24 h the medium replaced and the cultures further incubated for 72 h. The changes in the size of the cell population were measured by using the MTT-assay as reported previously and the IC_{50} values were calculated with the help of oGraphPad Perism.[7]

[a] Absorbance: cell population on the basis of MTT test.

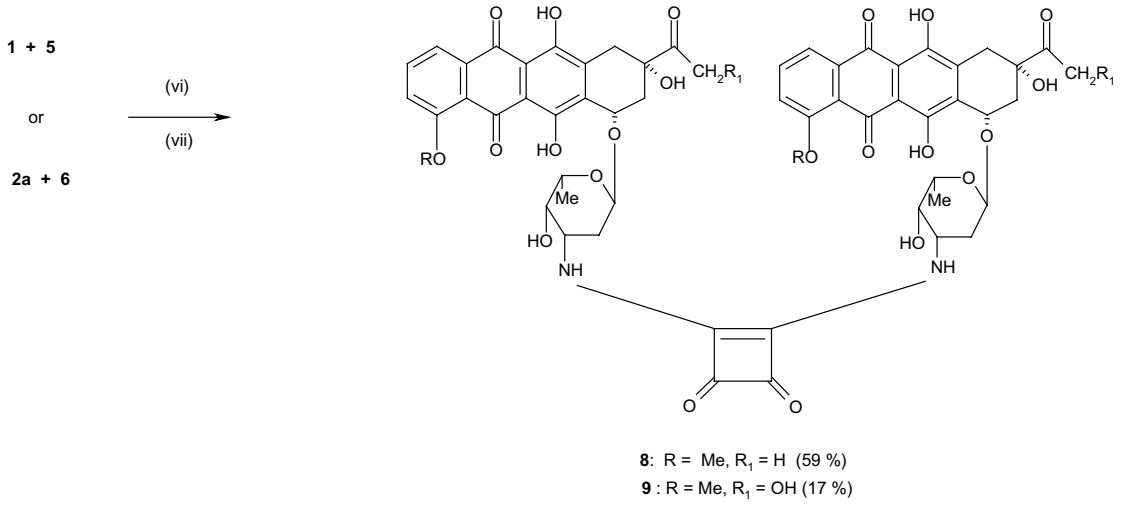
of the antibiotics **11** and **12** was carried out in practically the same manner. The development of the dimers is corroborated by the lack of the OMe signal of the squaric moiety and/or detection of the characteristic aliphatic chain of the linker (Table 2).

1. Biological activity and results

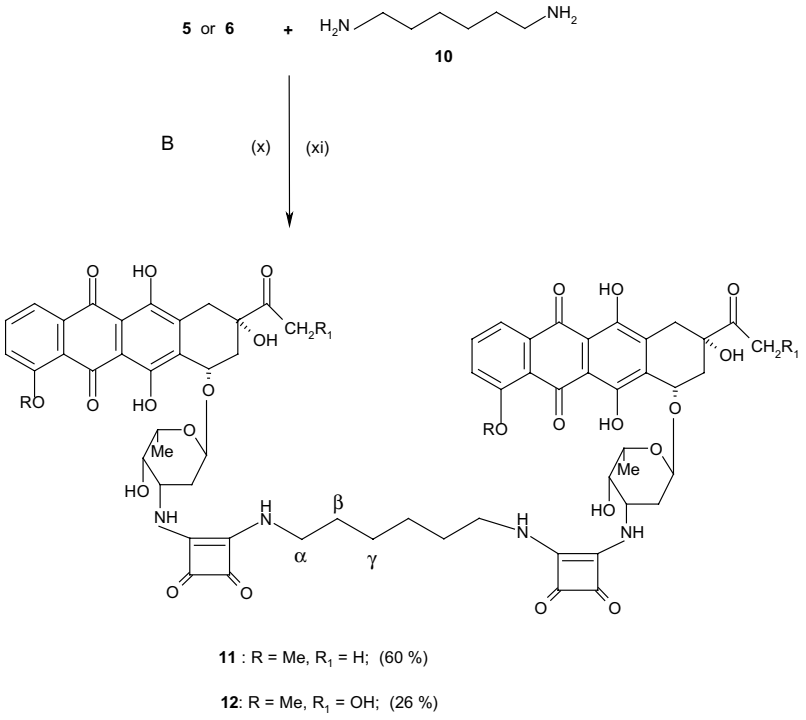
In the investigated cell line epirubicin (**2b**; Farmorubicin), applied as the control material, was more active (ca. 15 times) than carminomycin (**3**) (Table 3). The daunomycin squaric acid amide ester **5**, and the corresponding adriamycin derivative **6** possess IC_{50} values higher than 300 ng/mL. At the same time, the squaric acid amide ester of carminomycin (**7**) shows more pronounced inhibition of the cell proliferation than compounds **5** and **6**. Comparing the biological effects of the squaric acid diamides (**8** and **9**) derived from daunomycin and adriamycin, it was observed that the activity of **8** was much higher than that of **9** (IC_{50} 108.9 vs >300).

When the molecules of **5** or **6** were linked with hexamethylenediamine into the dimers **11** and **12**, respectively, again the daunomycin dimer (**11**) was more efficient than the corresponding adriamycin dimer (**12**).

These data show that the presence of the unsubstituted C-4 phenolic hydroxyl group of compound **7** is



Scheme 2. Formation of the covalent dimers of daunorubicin and doxorubicin. Reagents and conditions: (vi) EtOH, Et_3N (pH ~ 8.0), 20 °C, 48 h–1 week; (vii) column chromatography: Kieselgel 60 (0.063–0.20 mm, Merck), $CHCl_3$–MeOH (95:5) and/or $CHCl_3$–MeOH–HCOOH (9:1:0.05).



Scheme 3. Reaction of the daunomicinyl squaric acid monoester with α,ω-diaminoalkanes. Reagents and conditions: (x) EtOH, Et_3N (pH > 7.5–8.0), 48 h; (xi) column chromatography: Kieselgel 60 (0.062–0.20, Merck), $CHCl_3$–MeOH–HCOOH (9:1:0.05).

beneficial in the biological action, whereas the C-14 primary alcoholic hydroxyl group of **9** and **12** disadvantageously influences the cytotoxic activity.

Acknowledgements

The authors thank the Hungarian Academy of Sciences and the National Research Found (Grant OTKA to 46744, 42512 and 42567) for financial support, and Dr. Sándor Kéki (Department of Applied Chemistry, University of Debrecen) for recording the mass spectra and Dr. Marina Reznikova (Gause Institute of New Antibiotics, Moscow) for the HPLC measurements. The results included in this paper represent a part of the Ph.D. Theses of Anna Tevyashova.

References and notes

1. Arcamone, F. Doxorubicin anticancer antibiotics. Academic Press: New York, 1981; Vol. 17, p 1.

2. Young, C. W.; Wittes, R. E. In *Adriamycin: Its Expanding Role in Cancer Treatment*; Excerpta Medica: Tokyo, 1984, pp 479.
3. Sztaricskai, F.; Menyhárt, M.; Bognár, R. *Carbohydr. Res.* **1982**, *100*, C14–C16.
4. Menyhárt, M.; Sztaricskai, F.; Bognár, R. *Acta Chimica Hungarica* **1983**, *113*, 459.
5. Tietze, L. F.; Arlt, M.; Beller, M.; Glüsenkamp, K.-H.; Jähde, E.; Rajewsky, M. F. *Chem. Ber.* **1991**, *124*, 1215.
6. ACD/CNMR Predictor, version 6.00, *Advanced Chemistry Development*, Toronto, Canada, 1991. http://www.acdeabs.com.
7. Katona, Cs.; Timár, F.; Jeney, A.; Fischel, J. L.; Milano, G.; Pandi, E.; Kralovansky, J. *Anticancer Drugs* **1991**, *10*, 561.