SYNTHESIS AND CYTOSTATIC PROPERTIES OF DAUNORUBICIN DERIVATIVES, CONTAINING *N*-PHENYLTHIOUREA OR *N*-ETHYLTHIOUREA MOIETIES IN THE 3′-POSITION

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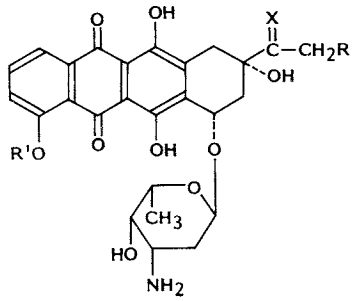
A series of phenylthiourea and ethylthiourea derivatives of daunorubicin and its congeners was prepared by reaction of the 3′-amino group of the antibiotic with phenylisothiocyanate or ethylisothiocyanate. *S*-Methylation yielded *S*-methylisothiouronium salts which when reacted with amines resulted in an intramolecular cyclization with the participation of the neighboring 4′-OH group. The structures and predominant conformations of the thiourea derivatives and daunorubicino(3′-*N*,4′-*O*-d)oxazolines were determined by ^{1}H and ^{13}C NMR. Cytostatic activities of the thiourea and oxazoline derivatives were compared with the cytostatic activities of *N*-methylurea and *N*-methyl-*N*-nitrosourea containing daunorubicin and its congeners. Carminomycin derivatives were endowed with the highest cytostatic activity.

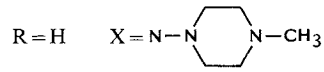
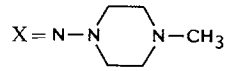
In the course of a screening program for novel second generation antitumor anthracycline antibiotics of daunorubicin (**1**) series, derivatives containing in 3′-position *N*-methylurea (**2**) or *N*-methyl-*N*-nitrosourea moieties (**3**) were synthesized[1]. In alkaline conditions, 3′-(*N*-methyl-*N*-nitrosoaminocarbonyl)daunorubicin (**3a**) or related compounds produce 3′-*N*,4′-*O*-carbonyl derivatives by intramolecular cyclization of intermediate 3-deamino-3′-isocyanato derivatives[1]. In this paper we report on the preparation of thiourea-containing derivatives of daunorubicin and its analogs which are susceptible to transformations with neighboring 4′-hydroxy group participation. Also, the structure-cytostatic activity relationship among urea and thiourea derivatives of anthracycline antibiotics was investigated.

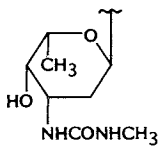
Chemistry

Daunorubicin (**1a**), carminomycin (**1b**), doxorubicin (**1c**) and 14-pimeloyloxydaunorubicin (**1d**)[1], upon interaction with phenylisothiocyanate or ethylisothiocyanate in pyridine afforded the corresponding phenylthiourea (**5a**～**5d**) or ethylthiourea (**6a** and **6c**) derivatives in 77～99% yield. Previously, **5a** was obtained from **1a** and phenylisothiocyanate in chloroform-methanol mixture in a yield of 64%[2]. *S*-Methylisothiouronium salts of these compounds **7a**, **7b** and **8a** were obtained by alkylation with CH_3I in methanol. Usually *S*-alkylisothiouronium salts easily produce guanidines by the action of amines. Compounds **7** and **8** were selected for their transformation to substituted guanidine derivatives (**9**). Upon interaction with primary amines (methylamine, *n*-pentylamine or tris(hydroxymethyl)aminomethane) **7a**, **7b** and **8a** yielded daunorubicino(3′,4′-d)oxazoline derivatives (**10a**, **10b** or **11a**), respectively. The formation of a 5-membered cycle is facilitated as in the case of transformations of *N*-methyl-*N*-nitrosoaminocarbonyl-derivatives of daunorubicin (**4**)[1].

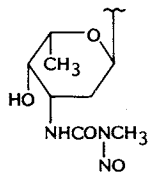
We could demonstrate by HPLC that storage of *S*-methylisothiouronium salt **7a** during 1-day in



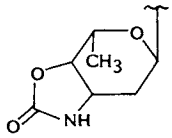
1a R′=CH_3 R=H X=O
1b R′=H R=H X=O
1c R′=CH_3 R=OH X=O
1d R′=CH_3 R=$OCO(CH_2)_5COOH$ X=O
1e R′=CH_3 R=$OCO(CH_2)_4COOH$ X=O
1f R′=CH_3 R=H X= 
1g R′=R=H X= 



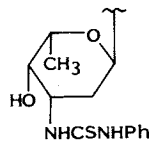
2a～2d, 2f, 2g



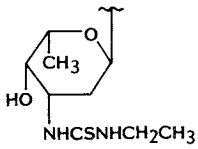
3a～3c



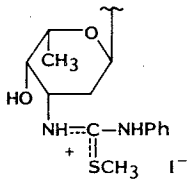
4a, 4b



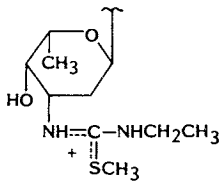
5a～5d



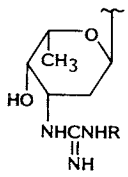
6a, 6c



7a, 7b



8a



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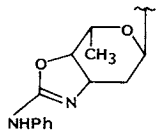


10a, 10b

methanol, resulted in the oxazoline derivative **10a**; the transformation of **7a** to oxazoline **10a** in acetonitrile proceeded at room temperature during one month. The interaction of primary amines (methylamine, *n*-pentylamine or tris(hydroxymethyl)aminomethane), with **7a** or **8a** led in all cases to the same products **10a** or **11a**, respectively. HPLC analysis of fresh methanolic solution of **7a** after addition of methanolic ammonia demonstrated the presence of **7a** and **10a**. Storage of this solution led to complete transformation of **7a** to **10a**.

The structures assigned to the thiourea derivatives **5** and **6** and the oxazoline-containing antibiotics **10** and **11** were supported by ^{13}C and ^{1}H NMR. In the Table 1 selected ^{1}H NMR data for thiourea and oxazoline derivatives are listed. Conformation of the carbohydrate ring in compounds **5** and **6** is similar to that of the parent antibiotics **1** ($_4C^1$). Carbon resonance assignments have been made from single-resonance spectra ^{13}C and 2D-^{13}C-^{1}H shift correlated spectroscopy *via* direct and long-range C-H couplings.

S-Methylthiouronium salts of teicoplanin in alkaline conditions gave rise to an intramolecular cyclization with the formation of both oxazoline- and imidazolone derivatives[3], but in our case the imidazolone compound **12** was not formed. Significant differences of chemical shifts of C-4′ and C-3′ carbons (up to 20 ppm) in the cyclization products fit more closely to structures **10a** and **11a**, and exclude the possibility of imidazolone **12** formation.

The cyclic structure of **10a** and **11a** was confirmed by selective double resonance ^{13}C-^{1}H. Coupling constant $J=3$ Hz between carbon C-NHPh and 4′-H demonstrated that spin coupling proceeds through 3 bonds, but not 4 bonds, as it would have been if the oxazoline structure was not closed.

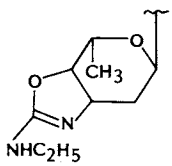
The structures **10** and **11** are possibly in tautomeric equilibria with the structure **13** (**10(11)⇄13**). Comparison of chemical shifts of carbon atoms of the phenyl ring with the data for corresponding model compounds, containing this fragment in amino or imino-forms[4,5], indicates that the amino-tautomer is predominant for **10a** in $CDCl_3$ and for **11a** in $CDCl_3$-CD_3OD solution.

^{1}H NMR data demonstrated that incorporation of C-3′ and C-4′ atoms into a 5-membered cycle leads to change of conformation $_4C^1$ (**14**) of the daunosamine cycle (Table 1). Coupling constants $J_{2'b,3'}=2.3$ Hz and $J_{2'a,3'}=3.8$ Hz indicate the absence of *trans*-diaxial coupling of these protons, which means that the 3′-H proton is equatorial. The large $J_{3',4'}=9.6$ Hz is possibly connected with a decrease of dihedral angle 3′-H~4′-H due to cyclization. Similar findings have been described for another

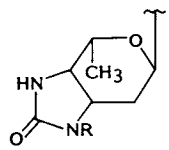
Table 1. ^{1}H NMR assignments of signals of sugar moieties of compounds **5a**~**5d**, **6a**, **6c**, **10a**, **10b** and **11a** in $CDCl_3$ (δppm) with $CHCl_3$ internal reference (7.25) and spin coupling constants (Hz).

Compound	Chemical shifts							Coupling constants						
	1′-H	2′-H_a	2′-H_b	3′-H	4′-H	5′-H	5′-CH_3	1′,2′b	1′,2′a	2′a,2′b	2′b,3′	2′a,3′	3′,4′	4′,5′
5a	5.49	2.06	1.68	4.73	3.38	4.25	1.27	3.9	<1	13.3	13.3	5.2	2.7	0.7
5b	5.45	2.13	1.69	4.76	3.77	4.26	1.28	4.1	<1	13.0	13.0	5.1		<1
5c	5.50	2.06	1.67	4.73	3.77	4.18	1.27	3.9	0.9	13.3	13.3	5.0	2.6	0.9
5d[a]	5.37	1.84	1.64	4.50	3.58	4.09	1.16	3.5	<1	13.4		5.2		<1
6a	5.49	2.02	1.78	4.65	3.78	4.24	1.29	3.8	<1	13.2	13.2	4.8	2.7	<1
6c[a]	5.44	1.85	1.76	4.46	3.65	4.12	1.23	3.9	<1	13.2	13.2	5.0		<1
10a	5.51	1.68	2.48	4.49	4.52	4.07	1.36	5.9	8.3	15.3	2.3	3.8	9.6	1.2
10b	5.49	1.69	2.50	4.50	4.50	4.05	1.37	5.9	8.0					
11a	5.56	1.69	2.52	4.52	4.95	4.17	1.38	6.3	7.8	16.0	2.2	3.5	9.5	1.5

[a] Small amounts of CD_3OD were added.



11a



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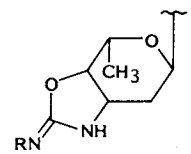

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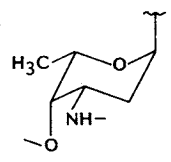

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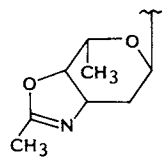
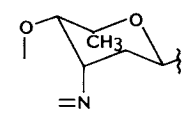


15a



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3′,4′-cyclo derivative of daunorubicin (**15**)[6] as well as for 3′-*N*,4′-*O*-carbonyl derivatives of these antibiotics (**4**)[1]. It suggests that cyclic derivatives of this type are in distorted conformation $_1C^4$ (**16**) or in twist-boat conformation. It is interesting to note that the coupling constants of the sugar protons in the compounds **10**, **11**, **12** and **15** are rather close to the coupling constants of the sugar protons of the doxorubicin base in CD_3COCD_3 solution[7]. It means that the relative distribution of the various forms of these cyclic compounds can be similar to that of the natural antibiotics.

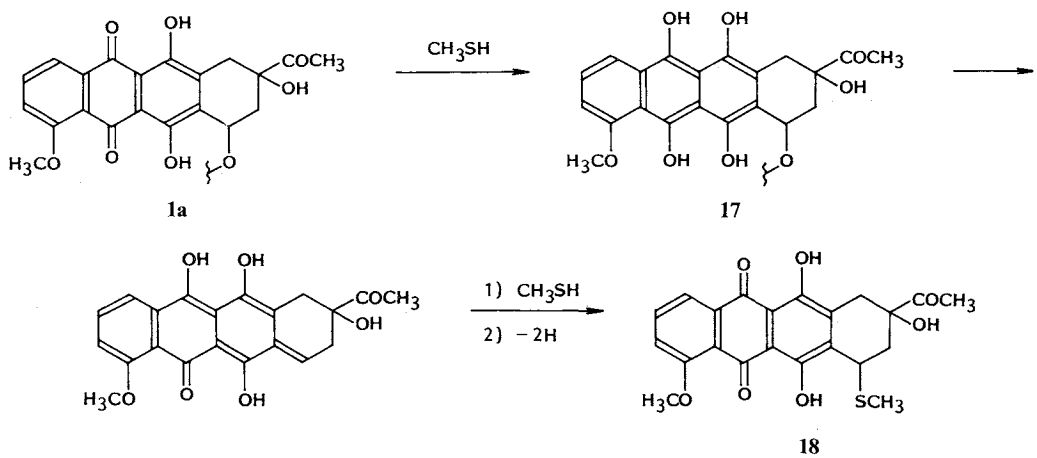
In the IR spectra of the compounds **10a**, **10b** and **11a** neither the frequency of an imidazolone CO group at 1705 cm^{-1} nor the absorption at 1735 cm^{-1} characteristic for urea[1] is present. An absorption peak at 1680 cm^{-1} demonstrated the presence of C=N group in these compounds.

Akin to the oxazolinone compounds **4**[1], the derivatives **10a**, **10b** and **11a** demonstrated $[\alpha]_D$ values that were very different from the starting compounds **1**, **5** and **6** (Table 2).

Table 2. Preparation and physical properties of compounds **5a**~**5d**, **6a**, **6c**, **10a**, **10b** and **11a**.

Compound	Yield (%)	MP (°C)	$[\alpha]_D$ (°)	Calcd C	Calcd H	Calcd N	Calcd S	Molecular formula	Found C	Found H	Found N	Found S	Rf	IR (KBr) cm^{-1}
5a	96.4	162	+ 82[a]	60.00	5.33	4.11	4.70	$C_{34}H_{34}N_2SO_{10}\cdot H_2O$	60.37	5.35	4.00	4.85	0.51	1530, 1500, 1230
5b	80.5	160	+146[c]	59.45	5.14	4.20	4.81	$C_{33}H_{32}N_2SO_{10}\cdot H_2O$	59.57	5.04	4.04	4.83	0.47	1535, 1500, 1246
5c	98.7	128	+102[c]	57.86	5.28			$C_{34}H_{34}N_2SO_{11}\cdot 1\frac{1}{2}H_2O$	57.94	5.34			0.45	1540, 1500, 1240
5d	76.6	144	+145[b]	57.47	5.65	3.27		$C_{41}H_{44}N_2SO_{14}\cdot 2H_2O$	57.43	5.41	3.39		0.44	1548~1530, 1505, 1240
6a	92.1	164	+269[a]	57.77	5.66	4.49		$C_{30}H_{34}N_2SO_{10}\cdot \frac{1}{2}H_2O$	57.74	5.74	4.22		0.50	1550~1540, 1230
6c	92.1	188	+207[a]	53.33	5.82	4.14		$C_{30}H_{34}N_2SO_{11}\cdot 2\frac{1}{2}H_2O$	53.14	5.72	3.92		0.34	1560~1550, 1249
10a	63.6	157	−260[a]			4.45		$C_{34}H_{33}N_2O_{10}$			4.27		0.55	1680, 1500, 1235
10b	58.4	158	−265[b]										0.54	1680, 1500, 1240
11a	52.2	166	− 66[a]	58.43	5.56	4.54		$C_{30}H_{32}N_2O_{10}\cdot 2H_2O$	58.10	5.63	4.29		0.24	1680, 1230

[a] (*c* 0.05, $CHCl_3$). [b] (*c* 0.01, $CHCl_3$). [c] (*c* 0.05, $CHCl_3$-CH_3OH, 9:1).



We attempted to obtain a 3'-guanidine-containing derivative of daunorubicin (**9**) by the interaction of the antibiotic **1a** (as base) with *S*-methylisothiouronium methylsulfate in the presence of alkali. 7-Deoxy-7-methylthiodaunomycinone (**18**) was isolated by TLC in a yield of 14%. It suggests that MeSH produced from *S*-methylisothiouronium salt reduced the anthraquinone nucleus of **1a** with the formation of the leuco derivatives **17**; cleavage of the glycosyl bond and attachment of a second molecule of methylmercaptane with following oxidation afforded **18** in a reaction sequence similar to that proposed for the interaction of **1a** with $Na_2S_2O_4$[8].

Cytostatic Activity

The inhibitory activity of the thiourea (**5** and **6**) and oxazoline (**10**) derivatives on the proliferation of murine leukemia cells L1210, human B-lymphoblast cells (Raji) and T-lymphoblast cells (Molt-4F) were compared with the inhibitory activity of the *N*-methylurea (**2**), *N*-methyl-*N*-nitrosourea (**3**) and oxazolone (**4**) derivatives described previously[1]. The IC_{50} of the test compounds are presented in Table 3. The most potent cytostatic agent was carminomycin **1b**; all derivatives of **1b** (*i.e.*, compounds **2g**, **3b** and **4b**) were ten to one hundred times more active than the corresponding derivatives of daunorubicin (**1a**) (*i.e.*, compounds **2a**, **2f**, **3a**, **4a**, **5a**, **6a** and **10a**). Doxorubicin (**1c**) and some of its derivatives **2c** and **5c** were considerably less inhibitory to tumor cell proliferation than daunorubicin **1a** and its derivatives **2a** and

Table 3. Inhibitory effects of urea and thiourea derivatives of daunorubicin and related compounds on the proliferation of murine leukemia L1210, human B-lymphoblast (Raji) and human T-lymphoblast (Molt-4F) cells, and their inhibitory effects on HIV-1-induced cytopathogenicity in T-lymphocyte (MT-4) cells.

Compound	Inhibition of tumor cell proliferation			Inhibition of HIV-1 replication in MT-4 cells	
	L1210	Raji	Molt-4F		
	IC_{50}[a] (μM)			CC_{50}[b] (μM)	EC_{50}[c] (μM)
1a*	0.04	0.017	0.044		
1b*	0.005	0.002	0.005		
1c*	0.367	0.043	0.056		
2a	0.556	0.488	0.577		
2c	6.217	4.017	6.25		
2d	3.98	1.858	4.362		
2e	2.99	1.011	3.805		
2f*	1.76	1.26	1.71		
2g*	0.037	0.018	0.041		
3a	1.631	0.538	0.509		
3b	0.045	0.041	0.047		
3c	0.636	0.180	0.437		
4a	1.40	0.733	0.655	3.25	>1.80
4b	0.077	0.042	0.072	0.313	>0.19
5a	0.702	0.507	0.737		
5c	1.127	0.432	0.748		
5d	0.526	0.361	0.755		
6a	0.293	0.084	0.280	0.379	>0.16
6c	0.347	0.049	0.284	0.248	>0.16
10a	1.65	0.392	0.838	0.386	>0.16

[a] IC_{50} required to inhibit tumor cell proliferation by 50%.
[b] Cytotoxic concentration required to reduce the viability of mock-infected MT-4 cells by 50%.
[c] Effective concentration of compound required to achieve 50% protection of MT-4 cells against the cytopathic effect of HIV-1.
* Asterisks indicate values for the antibiotic hydrochlorides.

5a. Conversion to alkali-soluble depot forms of doxorubicin (**2a**→**2d**, **2e**; **5a**→**5d**) or acid-soluble depot forms (**2g** and **2f**) did not result in an enhancement of the cytostatic activity. Comparison of **3a** with **4a** and **3b** with **4b** indicates that the cytostatic properties of the *N*-methyl-*N*-nitrosourea derivatives are not more pronounced than those of their degradation products which have no alkylating moieties. Only in doxorubicin series the presence of the cytotoxic group **3c** led to a 10-fold increase of the cytostatic activity (in comparison with **2c**).

As demonstrated previously, the structure-activity relationship of daunorubicin derivatives is quite different from that of carminomycin derivatives.

When evaluated on their inhibitory effects on human immunodeficiency virus (HIV), none of the compounds **4a**, **4b**, **6a**, **6c**, and **10a** showed anti-HIV-1 activity in human T-lymphocyte (MT-4) cells at subtoxic concentrations.

Experimental

Physico-chemical determinations were made on the following instruments: NMR; VXR-400 (Varian, U.S.A), EI-MS; Varian-MAT-112 spectrometer at 210~230°C ion source temperature and 70 eV electron energy, samples being introduced by direct insertion, IR spectra in KBr spectrophotometer SP-1100 (Pye Unicam, England), $[\alpha]_D$ determination; polarimeter Perkin-Elmer 241.

Analytical HPLC was performed on DuPont (U.S.A.) instrument 8800 equipped with a UV detector at 254 nm and Zorbax C8 (4.6×250 mm, 5 μm) column, flow rate 1 ml/minute at 37°C, mobile phases: (A) acetonitrile and 0.01 M orthophosphoric acid 30:70; (B) acetonitrile and 0.05 M of NaH_2PO_4 (pH 3.2) (30:70). Rt of **5a** 10.4 minutes, Rt of **6a** 7.3 minutes, Rt of **7a** 16.9 minutes, Rt of **8a** 13.8 minutes, Rt of **10a** 18.4 minutes, Rt of **11a** 13.7 minutes (A system), Rt of daunomycinone 5.89 minutes, Rt of **18** 7.44 minutes (B system).

TLC was carried out on Silufol plates (Kavalier, Czechoslovakia) in chloroform - benzene - methanol (10:1:2).

Properties of the compounds are presented in Table 2. The assays for measuring inhibition of tumor cell growth and anti-HIV-1 activity in MT-4 cells were performed as previously described[9,10].

Phenylthiourea Derivatives **5a**~**5d** and Ethylthiourea Derivatives **6a** and **6c**

To a solution of the antibiotic **1a**~**1d** (2 g) in 150 ml of dry pyridine phenylisothiocyanate (2.5 ml) or ethylisothiocyanate (3 ml) was added. The solution was stirred at room temperature for 24 hours (for phenylisothiocyanate) or 48 hours (for ethylisothiocyanate), then evaporated to a minimal volume *in vacuo* and the product (**5a**~**5d** or **6a** and **6c**) was precipitated with ether (200 ml). For analytical purposes the substances were purified by TLC.

S-Methylisothiouronium Salts **7a**, **7b** and **8a**

A solution of thiourea derivatives **5a**, **5b** or **6a** (600 mg) and 0.6 ml of MeI in dry methanol (70 ml) was stirred at room temperature for 2 hours (**5a** and **5b**) or 24 hours (**6a**). The reaction mixture was filtered, the filtrate was evaporated *in vacuo*, dissolved in chloroform (5 ml) and the product was precipitated by addition of hexane (50 ml), with yields of 70~85%.

Oxazoline Derivatives **10a**, **10b** and **11a**

To a solution of *S*-methylisothiouronium iodates **7a**, **7b** or **8a** (250 mg) in methanol (20 ml) *n*-pentylamine was added and the reaction was left overnight. The precipitate was collected by filtration, washed with methanol, dissolved in chloroform and washed with $NaHCO_3$ solution and then with water. After evaporation till minimum volume and addition of hexane, the crystalline oxazolines **10a**, **10b** or **11a** were obtained. For analysis the compounds were purified by TLC. Compound **10a** was purified by column chromatography (silica gel) upon elution with benzene, benzene - acetone (87:13) and methanol. The methanol fraction was evaporated and the product was obtained after precipitation with ether.

^{13}C NMR data for **10a** in $CDCl_3$: C-1 119.58, C-2 135.50, C-3 118.27, C-4 160.85, C-4a 120.70, C-5 186.54, C-5a 111.11 (or 111.05), C-6 156.33, C-6a 133.93, C-7 69.18, C-8 35.17, C-9 76.83, C-10 33.07, C-10a 134.46, C-11 155.66, C-11a 111.05 (or 111.11), C-12 186.40, C-12a 135.25, C-13 212.32, C-14 24.81, OCH_3 56.41, C-1′ 100.02, C-2′ 29.91, C-3′ 58.23, C-4′ 78.85, C-5′ 64.60, C-6′ 16.26, C(−NHPh) 157.03; phenyl ring: C *ipso* 139.60; C *orĭho* 119.33; C *meta* 129.00; C *para* 122.77 ppm.

^{13}C NMR data for **11a** in $CDCl_3$-CD_3OD: C-1 119.46, C-2 135.53, C-3 118.83, C-4 160.73, C-4a 120.48, C-5 186.68, C-5a 111.13 (or 111.00), C-6 155.80, C-6a 133.66, C-7 68.79, C-8 35.00, C-9 76.62, C-10 32.78, C-10a 134.15, C-11 155.22, C-11a 111.00 (or 111.13), C-12 186.42, C-12a 135.10, C-13 212.70, C-14 24.51, OCH_3 56.33; C-1′ 99.84, C-2′ 29.88, C-3′ 58.41, C-4′ 79.02, C-5′ 64.49, C-6′ 14.85, C($-NHC_2H_5$) 161.04, N−CH 37.25, N−CH_2−CH_3 14.65 ppm.

7-Deoxy-7-methylthiodaunomycinone (**18**)

Daunorubicin base was dissolved in water-propanol mixture (1:1). *S*-Methylisothiouronium methylsulfate (0.4 g) and 10% NaOH (2 ml) were added, and the reaction mixture was heated at 60°C for 2 hours (methylmercaptane smell was detected). The product was extracted with chloroform, the solution was washed with water and evaporated till minimal volume and precipitated with ether. By TLC in A system 0.06 g (14.8%) of compound **18** was isolated.

Anal Calcd for $C_{22}H_{20}O_7S$: C 61.68, H 4.70.
Found: C 61.66, H 4.78.

^1H NMR data ($CDCl_3$) δ 7.98 (1H, d, $J_{1,2}=7.7$ Hz, 1-H), 7.73 (1H, t, 2-H), 7.34 (1H, d, $J_{3,2}=8.4$ Hz, 3-H), 4.71 (1H, dd, $J_{7,8b}=3.8$ Hz and $J_{7,8a}=7.8$ Hz, 7-H), 4.06 (3H, s, OCH_3), 3.20 (1H, d, $J_{a,b}=15.2$ Hz, 10-H_b), 3.12 (1H, d, 10-H_a), 2.49 (1H, d, 8-H_b), 2.29 (1H, dd, $J_{a,b}=15.20$ Hz, 8-H_a), 2.45 (3H, s, SCH_3), 2.13 (3H, s, 14-H).

Mass spectrum: 428 (87.8%, $C_{22}H_{20}O_7S$), 382 (15.9%, M−SCH_3+H), 362 (16.7%, M−SCH_3−H_2O−H), 337 (100%, M−SCH_3−$COCH_3$−H), 383 (15.61%, M−$COCH_3$−2H).

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