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SYNTHESIS AND ANTICANCER PROPERTIES OF 7α-CHLORO-3-METHYL-1,1-DIOXOCEPH-3-EM-4-CARBOXYLIC ACID ESTERS

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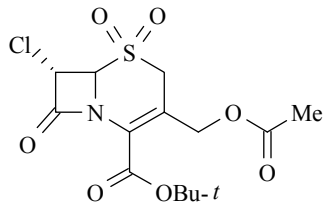
The tert-butyl esters of 3-azidomethyl-, 3-isocyanatomethyl-, 3-chloromethyl-, and 3-p-nitrophenylvinyl-7α-chloro-1,1-dioxoceph-3-em-4-carboxylic acid, and also esters of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid, and of 7α-chloro-3-methyl-2-dimethylaminomethylene-1,1-dioxoceph-3-em-4-carboxylic acid have been synthesized. Results of cytotoxic screening of these compounds in relation to cancer and normal cells in vitro are correlated and analyzed.

Keywords: esters of 7α-chloro-3-methyl-2-dimethylaminomethylene-1,1-dioxoceph-3-em-4-carboxylic acid, esters of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid, cytotoxic activity.

The directed structural modification of substituents in penicillin, cephalosporin, and 2-azetidinone carried out in the past 20 years has led to the discovery of compounds with anti-inflammatory, antiviral, anticancer, anticoagulant, and other activities "unplanned by nature". Their mechanism of action at the molecular level consists of the inhibition of specific serine- and cysteine-containing proteases as a result of acylation of hydroxyl or mercapto groups found in their active centers by the β-lactam ring [1].

The literature data referring to this investigation indicate that the structural variations of substituents in the β-lactam pharmacophore directed towards achieving effective inhibition of the target protease are accompanied by analogous, although less marked, effects on one or several related enzymes [2-5]. The negative side of this phenomenon consists of the probability of displaying undesirable secondary activity, but the positive is the possibility of using it for the targeted development of substances with new biological properties. Such an interpretation of the secondary activity of clavulanic acid ester, which is a specific inhibitor of the bacterial enzyme β-lactamase, in relation to Human Leucocyte Elastase (HLE) enabled the design of anti-inflammatory analogs of cephalosporin [6].

We encountered an analogous secondary effect on studying the biological properties of structural analogs of the *tert*-butyl ester of 7α-chloro-1,1-dioxoceph-3-em-4-carboxylic acid **1** and **2** [7].



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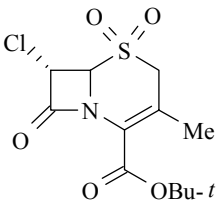

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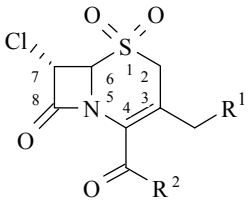

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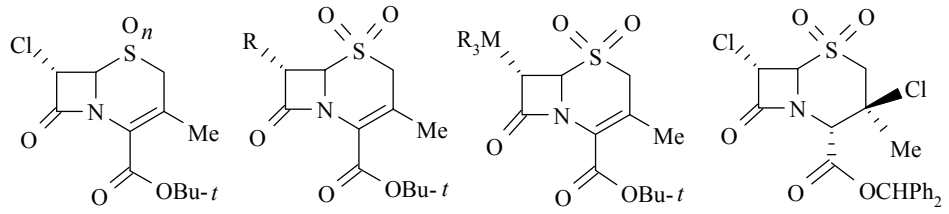
According to the data of Table 1 the presence of an acetoxymethyl group in position 3 of the cephem nucleus (in agreement with the data of [6]) provided the high inhibitory effect of compound **1** in relation to Porcine Pancreas Elastase (PPE) and a weak cytotoxic activity *in vitro* in relation to monolayer tumor cell lines HT-1080 (human fibrosarcoma) and MG-22a (mouse hepatoma). The deacetoxycephalosporin **2**, differing from cephalosporin **1** in the absence of the acetoxy group at position 3, was characterized by the reverse intensity of inhibitory and cytotoxic effects.

The substancial similarity of both compounds enabled a similar mechanism of action to be proposed for them at the molecular level, consisting of inhibition of the serine protease belonging to elastase family. In this way HLE is the main target for compound **1** [6]. The secondary targets are the specific elastases promoting growth and proliferation of cancer cells [8-10], and they are main for compound **2**. Consequently the bicyclic condensed system oxidized at position 1, and also substituted at positions 3, 4, and 7α by methyl, a carbonyl group, and halogen respectively, and represented by structure **3**, is a potential pharmacophore for anticancer activity.

In favor of this hypothesis evidence the negative results on testing the cytotoxic properties *in vitro* of resynthesized compounds **4a,b**, and also of the previously obtained structural analogs of cephalosporin **5-7** [7, 11, 12] with significant differences in the pharmacophore fragment of the molecule.



4 a,b **5 a,b** **6 a–c** **7**

4 a n = 0, **b** n = 1; **5 a** R = H, **b** R = I; **6 a** R = $SiEt_3$, **b** R = $SiMe_2Bu$-*t*, **c** R = $SiMe_2Ph$

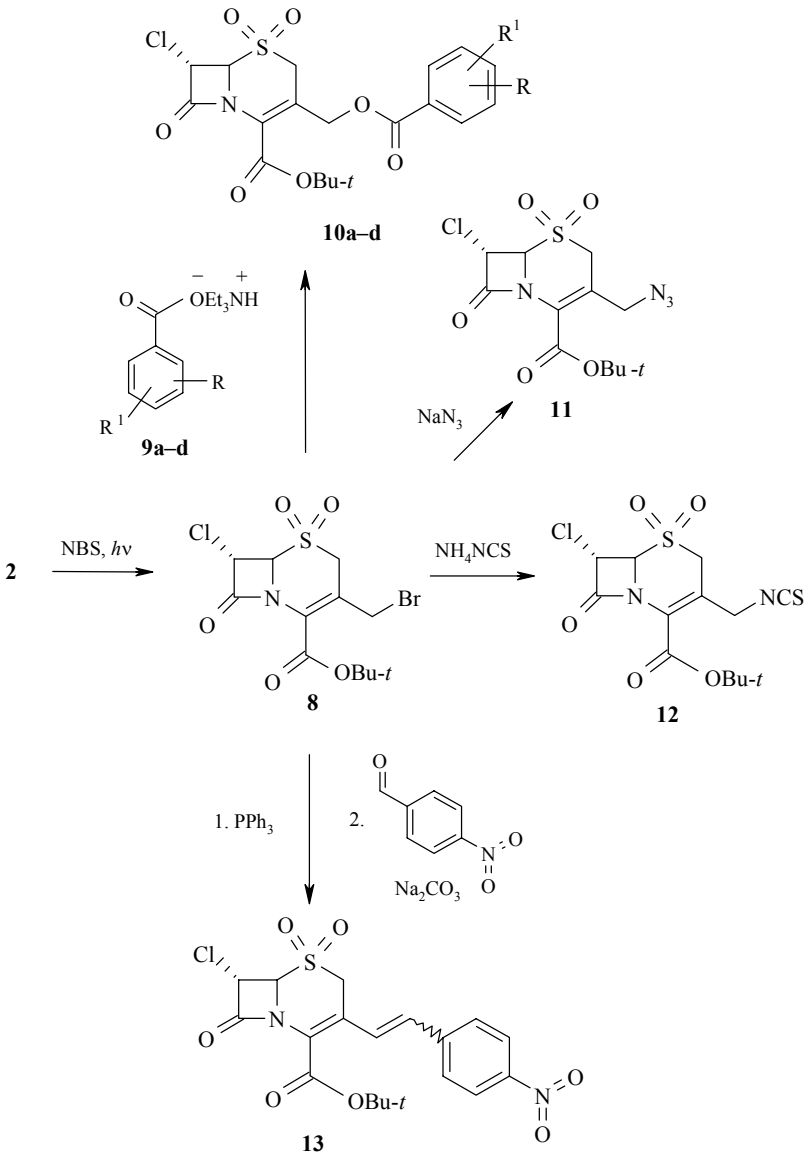
Thus a reduction in the degree of oxidation of the heterocyclic sulfur atom in **4a,b**, substitution of chlorine by hydrogen, iodine, or a trisubstituted silyl group in **5a,b**, **6a-c**, reduction of the double bond in the cephem nucleus and introduction of a chlorine atom at position 3 in compound **7** is accompanied by a significant decline or complete disappearance of the cytotoxic properties of these compounds in relation to cancer cells HT-1080 and MG-22A in comparison with compound **2**.

TABLE 1. Biological Properties of *tert*-Butyl Esters of 7α-Chlorocephalosporanic Acid Sulfone

Compound	Cytotoxic activity *in vitro*, LC_{50}, μg/ml *				IC_{50}, μmol
	HT-1080		MG-22A		
	CV	MTT	CV	MTT	
1	33	40	32	25	0.16±0.02
2	6	6	6	2	11±0,9

* LC_{50} is the concentration causing 50% cell death; CV staining with Crystal Violet; MTT staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; IC_{50} is the concentration causing 50% inhibition of the amidolytic activity of Porcine Pancreas Elastase in relation to the *p*-nitroanilide of N-methoxysuccinyl-Ala-Ala-Pro-Val as substrate.

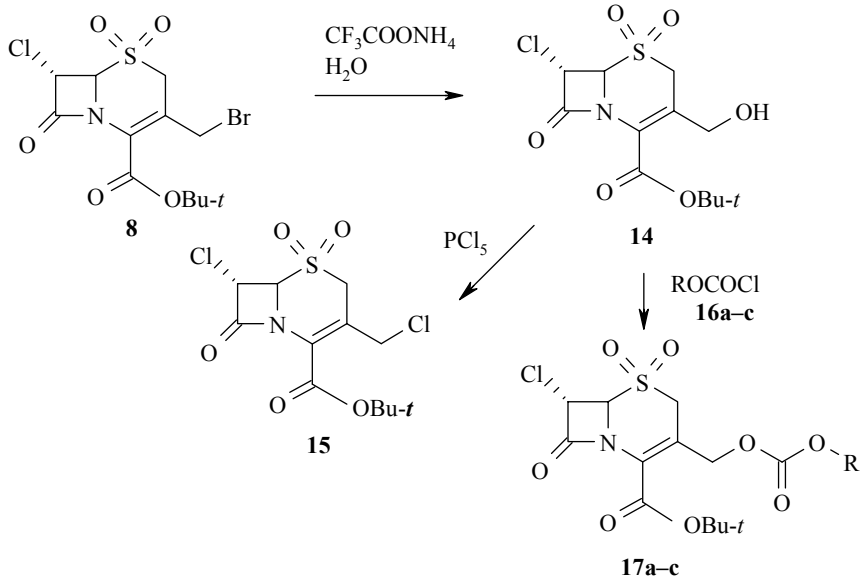
It proved to be more promising in this respect to substitute the methyl group in position 3 of the *tert*-butyl ester of 7α-chloro-1,1-dioxoceph-3-em-4-carboxylic acid (**2**). Synthesis of compounds of this type was effected from the *tert*-butyl ester of 3-bromomethyl-7α-chloro-1,1-dioxoceph-3-em-4-carboxylic acid (**8**), obtained by the allylic bromination of the methyl group in compound **2** [7]. Nucleophilic substitution of bromine in **8** by the triethylammonium salts of hydroxy- and acetoxy-substituted benzoic acids **9a-d** [13], by sodium azide or ammonium isothiocyanate, led to the preparation of the cephalosporin analogs **10a-d**, **11**, and **12**. The use of the Wittig reaction enabled introduction of a *p*-nitrobenzylidene substituent into the methyl group of compound **13**.



9,10 **a** R = 2-OH, R^1 = H; **b** R = 2-OH, R^1 = 4-OH; **c** R= 2-OAc, R^1 = H;
d R = 2-OAc, R^1 = 4-OAc

Exchange of the bromine in compound **8** by hydroxyl with ammonium trifluoroacetate with subsequent hydrolysis of the intermediate trifluoroacetoxy group led to the preparation of the 3-hydroxymethyl analog of cephalosporin **14**. With the aid of phosphorus pentachloride or chloroformates **14** was transformed into compounds **15** and **17** containing in position 3 of the cephem nucleus a methyl group substituted by chlorine or by a carbonate group respectively [14].

Data on the biological screening of compounds **8**, **10-15**, and **17**, given in Table 2, indicate that with the exception of compounds **8** and **10a**, they are characterized by moderate cytotoxicity in relation to cancer test cultures.



16,17 a R = CCl_3CH_2, **b** R = $BrCH_2CH_2$, **c** R = 4-$O_2NC_6H_4$

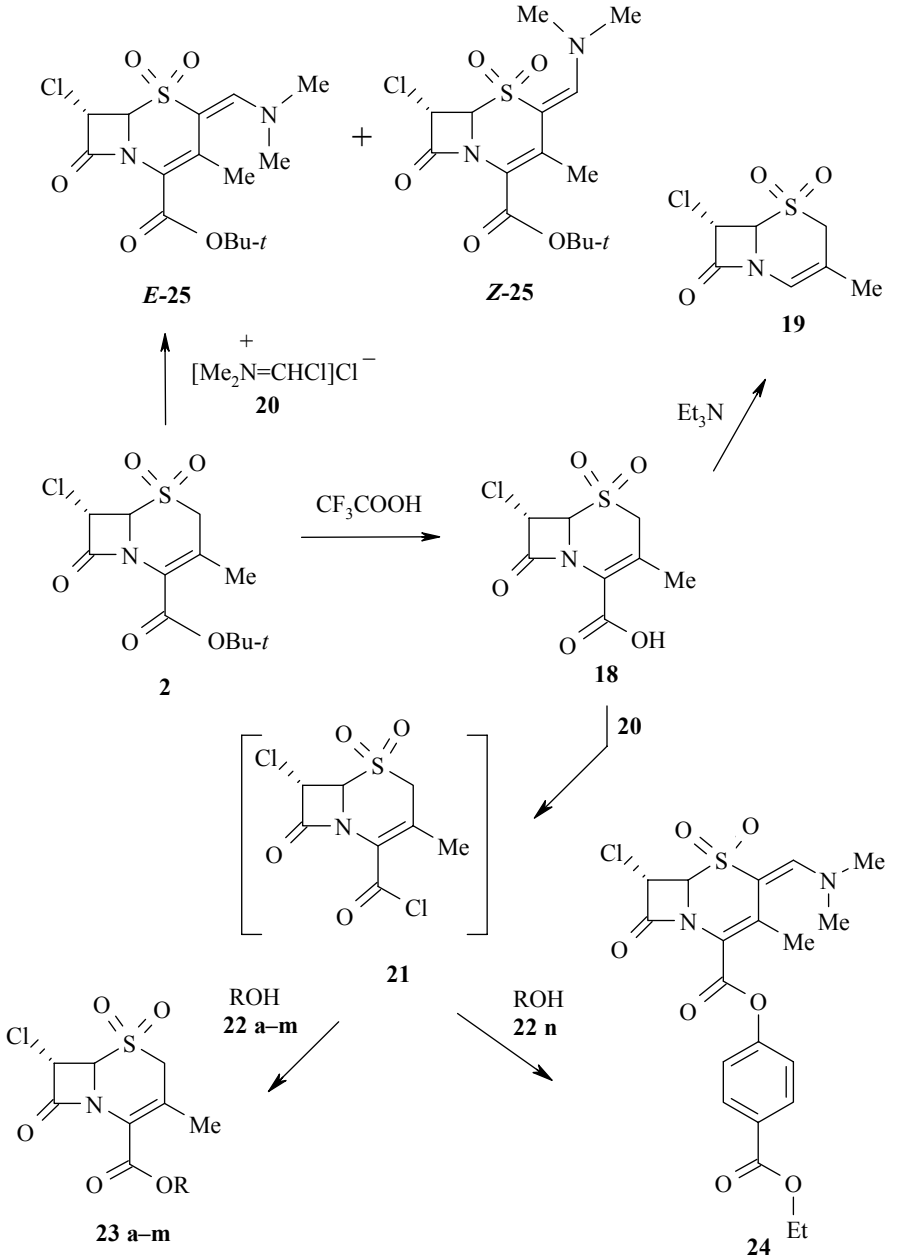
TABLE 2. Biological Activity of the *tert*-Butyl Ester of 7α-Chloro-1,1-dioxo-ceph-3-em-4-carboxylic Acid Modified in Position 3

Compound	Cytotoxic activity *in vitro*, LC_{50}, μg/ml				IC_{50}, μmol	References
	HT-1080		MG-22A			
	CV	MTT	CV	MTT		
8	12	5	6	1	—	[7]
10a	2	2	2	6	4.1 (41*)	[13]
10b	50	50	37	37	5.0 (68*)	[13]
10c	46	42	>50	>50	0.35	[13]
10d	52	53	31	40	15 (6.3*)	[13]
11	18	3	9	11	—	—
12	33	10	12	7	—	—
13	59	61	48	53	—	—
14	18	4	11	10	24	—
15	10	18	11	10	—	—
17a	39	52	46	58	13	[14]
17b	37	56	45	42	0.040	[14]
17c	53	72	45	62	0.047	[14]

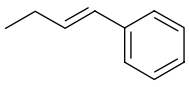
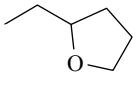
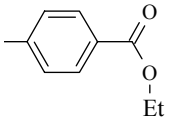
* Inhibition (%) of the amidolytic activity of Porcine Pancreas Elastase in relation to the *p*-nitroanilide of N-methoxysuccinyl-Ala-Ala-Pro-Val as substrate.

However the high inhibitory effect in relation to PPE displayed by individual representatives of this type of compounds (**10a**, **17b**, and **17c**) indicates that modification of the methyl group represents little promise since in principle it contributes to a reduction of the selectivity of anticancer action of this type of cephalosporin.

Modification of the ester group was therefore selected as the next subject of investigation of the dependence of structure and anticancer properties of of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid analogs.



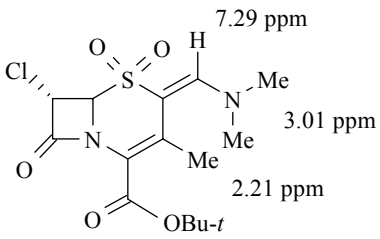
22, 23 a R = Me, **b** R = Et, **c** R = *i*-Pr, **d** R = *n*-Bu, **e** R = $CH_2CH{=}CH_2$, **f** R = CH_2CH_2Cl, **g** R = CH_2CCl_3, **h** R = *n*-C_5H_{11}, **i** R = *n*-C_7H_{15}, **j** R = Ph, **k** R = Bn,

l R =  , **m** R =  , **n** R = 

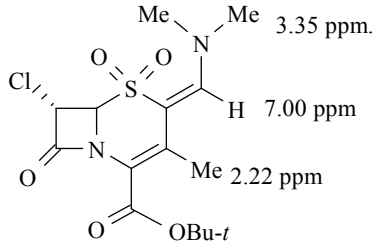
Esters of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid **23a-m** and also their analogs **24** and **25**, containing a dimethylaminomethylene group at position 2, were synthesized starting from the *tert*-butyl ester **2**. Its treatment with trifluoroacetic acid, as given in [15], led to the preparation of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid (**18**). Compound **18** is stable in acidic medium, but in neutral and in basic media it is subject to rapid decarboxylation with the formation of 7α-chloro-3-methyl-1,1-dioxoceph-3-em (**19**). The action of the Vilsmeier reagent **20** on the carboxyl group of cephem **18**, in a medium of methylene chloride according to [15], led to conversion into the acid chloride **21**, which without isolation was subjected to treatment with the appropriate alcohol. The object esters **23a-m** were isolated from the reaction mixture by column chromatography on silica gel. The use of the ethyl ester of *p*-hydroxybenzoic acid for esterification of **21** led to the preparation of ester **24**, having a dimethylaminomethylene group at position 2. The analogous reaction, based on the alkylating properties of the Vilsmeier reagent in relation to the cephem nucleus was noted in [16]. Treatment of *tert*-butyl ester **2** with this reagent led to the preparation of a product, substituted with dimethylaminomethylene in position 2, as a mixture of isomers *E*-**25** and *Z*-**25** in a ratio of 3 : 1.

The structures of isomers *E*-**25** and *Z*-**25** were determined by 2D-NOESY two-dimensional spectroscopy. The presence of a NOE between the dimethylamino and 3-methyl groups (indicating their closeness in space) and the absence of one between the 3-methyl group and the =CH proton in the predominant isomer confirms the *E*-orientation of the latter. In its turn, a NOE was observed in the minor isomer between the 3-methyl group and the =CH proton but was absent for the dimethylamino and 3-methyl groups thereby demonstrating the *Z*-orientation of the minor isomer.

The close disposition of the sulfone group leads to a displacement towards low field of the resonance signals of the neighboring dimethylamino group in the *Z*-**25** isomer and of the =CH proton in the *E*-**25** isomer.



E-**25**



Z-**25**

Biological screening of the synthesized esters of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid **23a-m**, **24**, and **25**, and the decarboxylated product **19** *in vitro* included determination of their cytotoxic properties in relation to monolayer cancer cell lines HT-1080 and MG-22A. Also assessed was their ability to initiate biosynthesis of nitric oxide radicals, the high reactivity of which is one of the components of the cytotoxic effect. Calculation of the specific NO-generating activity of compounds, consisted of determination of the NO ion concentration (nmol) in cellular medium in panel wells of volume 200 μliter after incubation for 72 h, in the presence of the substance being tested at a concentration of 50 μg/ml by the method of [18], then converting the value obtained to 100% of the amount of cells contained in the medium at the beginning of the experiment:

$$TG_{100} = G \cdot 100/C \text{ (nmol/μliter)}$$

where TG_{100} is the specific NO-generating activity of a compound; G the NO concentration (nmol) generated in the culture medium of volume 200 μliter by the surviving cells; C the percentage of surviving cells determined by CV-staining.

TABLE 3. Biological Activity of Esters of 7α-Chloro-3-methyl-1,1-dioxo-ceph-3-em-4-carboxylic Acid

Compound	Cytotoxic activity *in vitro*, LC_{50}, μg/ml					
	HT-1080			MG-22A		
	CV	MTT	TG_{100}	CV	MTT	TG_{100}
23d	28	16	135	18	22	350
23g	9	14	800	18	20	400
23h	17	23	1050	13	14	200
23i	32	15	450	28	24	250
23j	14	21	850	13	8	200
23k	25	20	340	12	16	350
23l	16	12	400	25	26	200
25a/25b	18	32	450	18	21	250

The tested esters may be divided into two groups according to the displayed anticancer effect. The *n*-butyl (**23d**), *n*-pentyl (**23h**), *n*-heptyl (**23i**), 2,2,2-trichloroethyl (**23g**), phenyl (**23j**), benzyl (**23k**), and cinnamyl (**23l**) esters, and also the isomeric mixture of *tert*-butyl esters *E*-**25**/*Z*-**25** substituted in position 2, which are characterized by LC_{50} values in the range 10-30 μg/ml and by active generation of nitric oxide radicals in the cell medium, fall into the first group (Table 3).

The second group of compounds, consisting of *tert*-butyl ester **2**, methyl, ethyl, isopropyl, allyl, chloroethyl, and furfuryl esters **23a-c**, **23e**, **23f**, **23m**, and also the *p*-ethoxycarbonylphenyl ester **24** substituted in position 2, which displayed a higher cytotoxic activity in relation to both or one of the cell cultures HT-1080 and MG-22A, were tested additionally in relation to B16 (mouse melanoma) and Neuro 2A (mouse neuroblastoma) cancer cells, and also normal cells 3T3 (mouse embryo fibroblasts) and BHK cells (golden hamster kidney fibroblasts) (Table 4).

Staining of 3T3 fibroblasts with Neutral Red (NR) enabled calculation of the value of the expected toxicity LD_{50} for the tested compounds, without resorting to experiments *in vivo*, with the aid of the equation [17]

$$\log LD_{50}\ (mg/kg) = 0.435 \times \log LC_{50}\ (mM) + 0.625$$

According to the data of Table 4 the methyl ester **23a** and the decarboxylated cephem **19** are characterized by a high cytotoxic effect in relation to both cancer cells and to normal cells. Such an absence of selectivity is reflected in the low LD_{50} values of 236 and 252 mg/kg for these compounds. An increase in the carbon chain in the ester group of compounds **23b**, **23c**, and **23e-g** to 2 or 3 carbon atoms enabled a strengthening of the toxic effect in relation to cancer and a weakening of it in relation to normal cells. The selectivity developed led to a more than twofold reduction in the toxicity index (LD_{50} 490 to 626 mg/kg). The leading position in effectiveness and selectivity of anticancer effect in this group belongs to *tert*-butyl ester **2** with LD_{50} 1162 mg/kg. The cytotoxic effect of compounds is connected with the intense generation of nitric oxide radicals by the cancer cells (see Table 4).

The results of the investigation carried out indicate that modification of the ester group in 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid is promising in the search for new anticancer substances permitting action both on the cytotoxic effectiveness of compounds and on their selectivity in relation to cancer and normal cells.

TABLE 4. Biological Activity of Esters of 7α-Chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic Acid

Com-pound	Cytotoxic activity *in vitro*, LC_{50}, μg/ml															LD_{50}, mg/kg
	HT-1080			MG-22A			B16			Neuro2A			BHK-21		3T3	
	CV	MTT	TG_{100}	CV	MTT	TG_{100}	CV	MTT	TG_{100}	CV	MTT	TG_{100}	CV	MTT	NR*	
2	6	6	200	6	2	450	3	2	633	2	2	500	nt**	nt	226	1162
23a	2.3	1	100	1	0.2	100	1.9	1	100	2	8	350	nt	nt	15	236
23b	3	1.9	700	4	4	400	13	27	200	11	16	850	31	32	35	490
23c	11	12	175	4	3	350	8	20	350	20	22	25	22	17	52	600
23e	2	3	300	3	0.2	300	3	4	300	11	21	300	nt	nt	58	626
23f	15	27	1000	5	1	200	13	27	350	22	14	83	32	32	31	495
23m	53	>100	27	0.3	2	450	1.5	1.1	800	0.7	0.8	175	2.5	2.0	55	659
24	4	5	200	0.4	0.3	150	12	26	100	32	21	100	17	27	nt	nt
19	2	2	650	1	2	150	2	2	250	2	2	550	2	1	11	252

* Staining with Neutral Red.

** Not tested.

EXPERIMENTAL

The ^{1}H NMR spectra were recorded on Bruker WH90/DS (90 MHz) and Varian Mercury 400 (400 MHz) spectrometers in $CDCl_3$, internal standard was TMS. Elemental analysis was carried out on a Carlo Erba 1108 analyzer and the difference of experimental and calculated values was ±0.4%. The IR spectra were taken on a Perkin-Elmer 580B spectrometer in nujol. A check on the progress of reactions was effected by TLC on Merck Kieselgel plates with visualization in UV light. The HPLC data were obtained on a Du-Pont Model 8800 instrument fitted with a UV detector (λ = 254 nm) and column (4.6×250 mm) packed with Symmetry C_{18} phase or Ultrasphere octyl in the system acetonitrile–water or acetonitrile–0.1 N phosphate buffer, pH 2.5 (60 : 40), rate 0.8-1.5 ml/min. Silica gel type Merck Kieselgel (0.063-0.230 mm) was used for preparative column chromatography. Reagents and materials from Acros, Aldrich, and Sigma were used in experiments. The two-dimensional 2D-NOESY spectra for compounds *E*-**25** and *Z*-**25** were recorded on a Varian Mercury 400 (400 MHz) spectrometer in $CDCl_3$ at 24°C using impulse gradient technology. When recording 2D-spectra a data matrix of size 4096×1024 was used, which provided τ_{2max} = 250 msec for ^{1}H when recording along the *F2* axis and τ_{1max} = 100 msec for ^{1}H along the *F1* axis. To optimize the signal-to-noise ratio the data matrix before Fourier transformation was supplemented with zeros twice and multiplied by the cosine function. The duration of the mixing time in 2D-NOESY was 1 sec. Optical density in the biological tests carried out in 96-well panels was determined with a Tetretek Multiscan MCC/340 horizontal spectrophotometer.

***tert*-Butyl Ester of 3-Azidomethyl-7α-chloroceph-3-em-4-carboxylic Acid Sulfone (11).** Sodium azide (18 mg, 0.25 mmol) was added to a solution of the *tert*-butyl ester of 3-bromomethyl-7α-chloroceph-3-em-4-carboxylic acid sulfone [6] (100 mg, 0.25 mmol) in DMF (10 ml). The mixture was stirred for 24 h at room temperature, then diluted with water (50 ml) and diethyl ether (50 ml). The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was evaporated at reduced pressure. The residue was fractionated on a chromatographic column of silica gel (eluent ethyl acetate–hexane, 1 : 3). Fractions with R_f 0.33 were combined and evaporated. Mp 124-126°C, yield 7 mg (8%). IR spectrum: 2100 (N_3), 1810 (β-lactam), 1720 cm^{-1} (C=O). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.55 (9H, s, C_4H_9); 3.77 and 4.06 (2H, two d, AB system, ^2J = 19, SO_2CH_2); 4.08 and 4.35 (2H, two d, AB system, ^2J = 15, CH_2N_3); 4.83 (1H, br. s, H-6); 5.33 (1H, d, ^3J = 1.5, H-7). Found, %: C 40.05; H 4.26; N 15.39. $C_{12}H_{15}ClN_4O_5S$. Calculated, %: C 39.73; H 4.17; N 15.44.

***tert*-Butyl Ester of 7α-Chloro-3-isothiocyanatomethylceph-3-em-4-carboxylic acid Sulfone (12).** Ammonium thiocyanate (28 mg, 0.30 mmol) was added to a solution of the *tert*-butyl ester of 3-bromomethyl-7α-chloroceph-3-em-4-carboxylic acid sulfone (100 mg, 0.25 mmol) in DMF (10 ml). The mixture was stirred for 24 h at room temperature, diluted with water (50 ml) and diethyl ether (50 ml). The organic phase was separated, and dried over anhydrous Na_2SO_4. The solvent was evaporated at reduced pressure. The residue was fractionated on a chromatographic column of silica gel (eluent was ethyl acetate–hexane, 1 : 3). Fractions with R_f 0.22 were combined and evaporated. The oily substance had >95% content of the main substance, according to HPLC analysis, yield 30 mg (32%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.55 (9H, s, C_4H_9); 3.62 and 4.44 (2H, two d, AB-system, ^2J = 14, CH_2NCS); 3.77 and 4.22 (2H, two d, AB system, ^2J = 19, SO_2CH_2); 4.84 (1H, m, H-6); 5.31 (1H, d, ^3J = 1.5, H-7). Found, %: C 41.48; H 4.09; N 7.28. $C_{13}H_{15}ClN_2O_5S_2$. Calculated, %: C 41.21; H 3.99; N 7.39.

Mixture of *tert*-Butyl Esters of *cis*-7α-Chloro-3-(4-nitrophenylvinyl)ceph-3-em-4-carboxylic Acid Sulfone and *trans*-7α-Chloro-3-(4-nitrophenylvinyl)ceph-3-em-4-carboxylic Acid Sulfone (13). *p*-Nitrobenz-aldehyde (17 mg, 0.11 mmol) and 5% Na_2CO_3 solution (5 ml) were added to a solution of the *tert*-butyl ester of 7α-chloro-3-(triphenylphosphonium)methylceph-3-em-4-carboxylic acid bromide [6] (30 mg, 0.045 mmol) in dichloromethane (10 ml). The mixture was stirred for 2 h at room temperature, washed with 5% $NaHSO_3$ solution (50 ml), and dried over Na_2SO_4. The solvent was evaporated at reduced pressure. The residue was fractionated on a chromatographic column of silica gel (eluent ethyl acetate–hexane, 1 : 1). Fractions with R_f 0.51 were combined and evaporated. Yield 10 mg (49%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): *cis*-isomer – 1.55

(9H, s, C_4H_9); 3.51 and 3.77 (2H, two d, AB system, ^2J = 19, SO_2CH_2); 4.80 (1H, br. s, H-6); 5.31 (1H, d, ^3J = 2, H-7); 6.62 and 6.82 (2H, two d, ^3J = 10, CH=CH); 7.48 (2H, d, ^3J = 9, C_6H_4); 8.24 (2H, two d, ^3J = 9, C_6H_4); *trans*-isomer – 1.64 (9H, s, C_4H_9); 4.06-4.26 and 3.77 (2H, m, AB system, ^2J = 17, SO_2CH_2); 4.86 (1H, m, H-6); 5.37 (1H, d, ^3J = 2, H-7); 6.53 and 7.86 (2H, two d, ^3J = 14, CH=CH); 7.60 and 8.26 (4H, two d, ^{3}J = 9, C_6H_4). Found, %: C 49.83; H 4.34; N 5.77. $C_{19}H_{19}ClN_2O_7S \cdot 0.25\ H_2O$. Calculated, %: C 49.67; H 4.27; N 6.09.

***tert*-Butyl Ester of 7α-Chloro-3-chloromethylceph-3-em-4-carboxylic Acid Sulfone (15).** Phosphorus pentachloride (50 mg, 0.19 mmol) was added to a solution of the *tert*-butyl ester of 7α-chloro-3-hydroxymethylceph-3-em-4-carboxylic acid sulfone [14] (50 mg, 0.15 mmol) in dichloromethane (20 ml) at 0°C. The mixture was stirred for 30 min at room temperature, diluted with dichloromethane (20 ml), the solution was washed with 5% Na_2CO_3 solution (40 ml), and dried over anhydrous Na_2SO_4. The solvent was evaporated at reduced pressure. The residue was fractionated on a chromatographic column of silica gel (eluent ethyl acetate–hexane, 1 : 4). Fractions with R_f 0.31 were combined and evaporated. Mp 142-144°C, yield 33 mg (62%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.66 (9H, s, C_4H_9); 3.75 and 4.17 (2H, two d, AB system, ^3J = 18, SO_2CH_2); 4.26 and 4.62 (2H, two d, AB system, ^2J = 12, CH_2Cl); 4.80 (1H, br. s, H-6); 5.28 (1H, d, ^3J = 1.5, H-7). Found, %: C 40.52; H 4.30; N 3.81. $C_{12}H_{15}Cl_2NO_5S$. Calculated, %: C 40.46; H 4.24; N 3.93.

7α-Chloro-3-methyl-1,1-dioxoceph-3-em (19). Trifluoroacetic acid (2 ml, 38 mmol) was added to a solution of the *tert*-butyl ester of 7α-chloro-3-methylceph-3-em-4-carboxylic acid (342 mg, 1.11 mmol) in dichloromethane (30 ml) at 0°C. The mixture was stirred for 1 h at room temperature, diluted with dichloromethane (100 ml), and with water (100 ml). The organic phase was separated, and extracted with 5% Na_2CO_3 solution (40 ml). The aqueous extract was acidified with conc. HCl to pH 2, and extracted with ethyl acetate. The extract was evaporated under reduced pressure. The residue was crystallized from diethyl ether. 7α-Chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid (**18**) (254 mg, 90%) was obtained and was dissolved in a mixture of acetone (20 ml) and triethylamine (2 ml). The solution was stirred at room temperature for 1 h, and the solvent was evaporated under reduced pressure. The residue was fractionated on a chromatographic column of silica gel (eluent ethyl acetate–hexane, 1 : 1). Fractions with R_f 0.48 were combined and evaporated. Mp 122-124°C, yield 195 mg (90%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.80 (3H, s, 3-CH_3); 3.48 and 3.96 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.73 (1H, m, H-6); 5.31 (1H, d, ^3J = 1.8, H-7); 6.45 (1H, m, H-4). Found, %: C 38.03; H 3.77; N 6.29. $C_7H_8ClNO_3S$. Calculated, %: C 37.93; H 3.64; N 6.32.

Preparation of Esters of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone and 7α-Chloro-2-(dimethylaminomethylene)-3-methylceph-3-em-4-carboxylic Acid Sulfone (23, 24) (Typical Procedure). A mixture of oxalyl chloride (98 μliter) and DMF (10μliter) was added with stirring at room temperature to a suspension of 7α-chloro-3-methylceph-3-em-4-carboxylic acid sulfone (100 mg, 0.375 mmol) in dichloromethane (20 ml). The reaction mixture was heated at 40°C for 20 min, cooled to room temperature, and concentrated at reduced pressure. The residue, containing the acid chloride (**21**) of 7α-chloro-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acid, was dissolved in dichloromethane (15 ml). A mixture consisting of alcohol **22** (0.75 mmol) and triethylamine (130 μliter, 0.93 mmol) was added to the obtained solution cooled to -5°C. The reaction mixture was stirred for 20 min, diluted with dichloromethane (20 ml), washed with saturated NH_4Cl solution (40 ml), and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was fractionated on a chromatographic column of silica gel, and esters **23**, **24** were obtained.

Methyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23a) was obtained by the typical procedure using methanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.42. Mp 159-161°C, yield 24 mg (23%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.11 (3H, s, 3-CH_3); 3.54 and 3.97 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 3.88 (3H, s, OCH_3); 4.73 (1H, m, H-6); 5.28 (1H, d, ^3J = 1.5, H-7). Found, %: C 38.81; H 3.69; N 4.91. $C_9H_{10}ClNO_5S$. Calculated, %: C 38.65; H 3.60; N 5.01.

Ethyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Ester Sulfone (23b) was obtained by the typical procedure using ethanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.52. Mp 106-107°C, yield 64 mg (58%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.33 (3H, t, ^3J = 7, CH_3); 2.09 (3H, s,

3-CH_3); 3.68 and 3.97 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.28 and 4.44 (2H, two q, ^3J = 7, CH_2); 4.77 (1H, m, H-6); 5.29 (1H, d, ^3J = 1.5, H-7). Found, %: C 41.01; H 4.21; N 4.71. $C_{10}H_{12}ClNO_5S$. Calculated, %: C 40.89; H 4.12; N 4.77.

Isopropyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23c) was obtained by the typical procedure using 2-propanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0,60. Mp 161-163°C, yield 57 mg (50%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.44 (6H, d, ^3J = 6, $(C\underline{H}_3)_2CH$); 2.08 (3H, s, 3-CH_3); 3.66 and 4.00 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.80 (1H, m, H-6); 5.17 (1H, m, ^3J = 6, $Me_2C\underline{H}$); 5.26 (1H, d, ^3J = 1.5, H-7). Found, %: C 43.04; H 4.67; N 4.41. $C_{11}H_{14}ClNO_5S$. Calculated, %: C 42.93; H 4.59; N 4.55.

***n*-Butyl ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23d)** was obtained by the typical procedure using *n*-butanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.70. Mp 81-83°C, yield 46 mg (38%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 0.93 (3H, t, ^3J = 6, $(CH_2)_3C\underline{H}_3$) 1.55-1.88 (4H, m, $2CH_2$); 2.09 (3H, s, 3-CH_3); 3.69 and 4.00 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.31 (2H, t, ^3J = 6, CH_2); 4.77 (1H, m, H-6); 5.27 (1H, d, ^3J = 1.5, H-7). Found, %: C 44.90; H 5.11; N 4.24. $C_{12}H_{16}ClNO_5S$. Calculated, %: C 44.79; H 5.01; N 4.35.

Allyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23e) was obtained by the typical procedure using allyl alcohol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.70. Mp 128-130°C, yield 55 mg (48%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.11 (3H, s, 3-CH_3); 3.66 and 4.00 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.80-4.83 (2H, m, $C\underline{H}_2CH{=}CH_2$); 4.88 (1H, m, H-6); 5.27 (1H, d, ^3J = 2, H-7); 5.27 (1H, d, *J* = 11, *cis*-CH=C$\underline{H}_2$); 5.44 (1H, d, *J* = 18, *trans*-CH=C$\underline{H}_2$); 5.89 (1H, ddt, *J* = 18, *J* = 11, *J* = 6, C$\underline{H}$=CH_2). Found, %: C 43.38; H 4.11; N 4.42. $C_{11}H_{12}ClNO_5S$. Calculated, %: C 43.21; H 3.96; N 4.58.

2-Chloroethyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23f) was obtained by the typical procedure using 2-chloroethanol and was isolated from fractions (eluent ethyl acetate–hexane 1 : 1) with R_f 0.75. Mp 136-138°C, yield 65 mg (53%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.11 (3H, s, 3-CH_3); 3.71 and 3.95 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 3.81 (2H, t, ^3J = 6, $CH_2C\underline{H}_2Cl$); 4.45 and 4.60 (2H, two t, ^3J = 6, $C\underline{H}_2CH_2Cl$); 4.80 (1H, m, H-6); 5,31 (1H, d, ^3J = 2, H-7). Found, %: C 36.74; H 3.45; N 4.21. $C_{10}H_{11}Cl_2NO_5S$. Calculated, %: C 36.60; H 3.38; N 4.27.

2,2,2-Trichloroethyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23g) was obtained by the typical procedure using 2,2,2-trichloroethanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 3) with R_f 0.48. Mp 168-170°C, yield 74 mg (50%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.20 (3H, s, 3-CH_3); 3.73 and 4.00 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.80 (1H, m, H-6); 4.82 and 5.29 (2H, two d, AB system, ^2J = 12, CH_2CCl_3); 5.20 (1H, d, ^3J = 2, H-7). Found, %: C 30.39; H 2.35; N 3.43. $C_{10}H_9Cl_4NO_5S$. Calculated, %: C 30.25; H 2.28; N 3.53.

***n*-Pentyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23h)** was obtained by the typical procedure using *n*-pentanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.58. Mp 62-63°C, yield 65 mg (52%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 0.91 (3H, t, ^3J = 6, $(CH_2)_4C\underline{H}_3$); 1.17-1.91 (6H, m, $CH_2(C\underline{H}_2)_3CH_3$); 2.13 (3H, s, 3-$CH_3$); 3.68 and 3.97 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.28 (2H, t, ^3J = 6, $C\underline{H}_2C_4H_9$); 4.75 (1H, m, H-6); 5.29 (1H, d, ^3J = 2, H-7). Found, %: C 46.72; H 5.56; N 4.11. $C_{13}H_{18}ClNO_5S$. Calculated, %: C 46.50; 5.40; N 4.17.

***n*-Heptyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23i)** was obtained by the typical procedure using *n*-heptanol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.58. Mp 92°C, yield 61 mg (45%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 0.84 (3H, t, ^3J = 6, $CH_2(CH_2)_5C\underline{H}_3$); 1.00-1.75 (10H, m, $CH_2(C\underline{H}_2)_5CH_3$); 2.11 (3H, s, 3-$CH_3$); 3.69 and 3.98 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.26 (2H, t, ^3J = 6, $C\underline{H}_2(CH_2)_5CH_3$); 4.77 (1H, m, H-6); 5.11 (1H, d, ^3J = 2, H-7). Found, %: C 49.82; H 6.29; N 3.71. $C_{15}H_{22}ClNO_5S$. Calculated, %: C 49.51; H 6.09; N 3.85.

Phenyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23j) was obtained by the typical procedure using phenol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.33.

Mp 141-143°C, yield 20 mg (15%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.17 (3H, s, 3-CH_3); 3.73 and 4.04 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.84 (1H, m, H-6); 5.37 (1H, d, ^3J = 2, H-7); 7.15-7.57 (5H, m, C_6H_5). Found, %: C 49.31; H 3.60; N 4.07. $C_{14}H_{12}ClNO_5S$. Calculated, %: C 49.20; H 3.54; N 4.10.

Benzyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23k) was obtained by the typical procedure using benzyl alcohol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.80. Mp 143-145°C, yield 60 mg (42%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.06 (3H, s, 3-CH_3); 3.66 and 3.93 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.73 (1H, m, H-6); 5.01 and 5.24 (2H, two d, AB system, ^2J = 12, CH_2Ph); 5.31 (1H, d, ^3J = 2, H-7); 7.40 (5H, m, C_6H_5). Found, %: C 50.69; H 4.02; N 3.8. $C_{15}H_{14}ClNO_5S$. Calculated, %: C 50.64; H 3.97; N 3.94.

Cinnamyl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23l) was obtained by the typical procedure using cinnamyl alcohol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.66. Mp 130-132°C, yield 69 mg (48%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 2.08 (3H, s, 3-CH_3); 3.62 and 3.95 (2H, two d, AB system, ^2J = 18, SO_2CH_2); 4.75 (1H, m, H-6); 4.93 (2H, d, ^3J = 5, C<u>H</u>$_2$CH=CH); 5.23 (1H, d, ^3J = 2, H-7); 6.31 (1H, dt, ^3J = 16, ^3J = 5, CH_2C<u>H</u>=CH); 6.75 (1H, d, ^3J = 16, CH_2CH=C<u>H</u>); 7.20-7.58 (5H, m, C_6H_5). Found, %: C 53.31; H 4.82; N 3.60. $C_{17}H_{18}ClNO_5S$. Calculated, %: C 53.19; H 4.73; N 3.65.

Tetrahydrofurfuryl Ester of 7α-Chloro-3-methylceph-3-em-4-carboxylic Acid Sulfone (23m) was obtained by the typical procedure using tetrahydrofurfuryl alcohol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.37. Mp 140-142°C, yield 11 mg (3%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.71-2.04 (6H, m, 3CH_2, C_4H_7O); 2.15 (3H, s, 3-CH_3); 3.55-3.73 (1H, m, 2-CH, C_4H_7O); 3.71 and 4.01 (2H, two d, AB system, ^2J = 19, SO_2CH_2); 4.15-4.31 (2H, m, $COOCH_2$); 4.84 (1H, m, H-6); 5.26 (1H, d, ^3J = 2, H-7). Found, %: C 43.07; H 4.32; N 4.07. $C_{12}H_{14}ClNO_6S$. Calculated, %: C 42.93; H 4.20; N 4.17.

4-Ethoxycarbonylphenyl Ester of 2*E*-7α-Chloro-2-(dimethylamino-methylene)-3-methylceph-3-em-4-carboxylic Acid Sulfone (24) was obtained by the typical procedure using 4-ethoxycarbonylphenol and was isolated from fractions (eluent ethyl acetate–hexane, 1 : 1) with R_f 0.08. Mp 173-175°C, yield 30 mg (17%). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.33 (3H, t, ^3J = 7, CH_3), 2.51 (3H, s, 3-CH_3); 3.21 (6H, s, $N(CH_3)_2$); 4.37 (2H, q, ^3J = 7, CH_2); 4.77 (1H, d, ^3J = 2, H-6); 5.24 (1H, d, ^3J = 2, H-7); 7.28 and 8.06 (4H, two d, ^3J = 9, C_6H_4); 7.31 (1H, s, =$CHNMe_2$). Found, %: C 50.82; H 4.53; N 5.65. $C_{20}H_{21}ClN_2O_7S$. Calculated, %: C 51.23; H 4.51; N 5.97.

Mixture of *tert*-Butyl Ester of 2*E*-7α-Chloro-2-(dimethylaminomethylene)-3-methylceph-3-em-4-carboxylic Acid Sulfone and *tert*-Butyl Ester of 2*Z*-7α-Chloro-2-(dimethylaminomethylene)-3-methylceph-3-em-4-carboxylic Acid Sulfone 25a and 25b. A mixture consisting of oxalyl chloride (162 μliter, 1.86 mmol) and DMF (48 μliter, 0.62 mmol) was added at room temperature in an atmosphere of argon to a stirred suspension of the *tert*-butyl ester of 7α-chloro-3-methylceph-3-em-4-carboxylic acid sulphone (200 mg, 0.65 mmol) in dichloromethane (20 ml). The reaction mixture was heated at 40°C for 20 min, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in dichloromethane (15 ml). Triethylamine (130 μliter, 0.93 mmol) was added to the obtained solution, the mixture was stirred for 20 min, diluted with dichloromethane (20 ml), washed with saturated NH_4Cl solution (40 ml), and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was fractionated on a chromatographic column of silica gel (eluent ethyl acetate–hexane, 2 : 1). Fractions with R_f 0.44 were combined and evaporated. Yield 134 mg (58%). According to the NMR spectrum (400 MHz) the substance is a mixture of *E*- and *Z*-isomers in a ratio of 3 : 1. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): *Z*-**25** – 1.53 (9H, s, C_4H_9); 2.22 (3H, s, 3-CH_3); 3.35 (6H, s, $N(CH_3)_2$); 4.55 (1H, d, ^3J = 1.5, H-6); 5.24 (1H, d, ^3J = 1.5, H-7); 7.02 (1H, s, =$CHNMe_2$); *E*-**25** – 1.53 (9H, s, C_4H_9); 2.21 (3H, s, 3-CH_3); 3.01 (6H, s, $N(CH_3)_2$); 4.47 (1H, d, ^3J = 1.5, H-6); 5.06 (1H, d, ^3J = 1.5, H-7); 7.29 (1H, s, =$CHNMe_2$). Found, %: C 47.99; H 5.75; N 7.40. $C_{15}H_{21}ClN_2O_5S$. Calculated, %: C 47.81; H 5.62; N 7.43.

Biological Screening. The cytotoxic properties of the synthesized substances were measured *in vitro* in relation to monolayers of cancer cells HT-1080 (human fibrosarcoma), MG-22A (mouse hepatoma), B16 (mouse melanoma), Neuro 2A mouse neuroblastoma) and of normal cells 3T3 (Swiss Albino mouse fibroblasts) and BHK

cells (golden hamster baby kidney fibroblasts), and also the concentration of nitric oxide radicals in Griess method was determined in 96-well plastic panels using CV, MTT, and NR dyestuffs in accordance with the procedures [18, 19] tested by us previously [7].

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