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Synthesis and Antitumor Activity of Selected 7-Alkylidene Substituted Cephems

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Abstract—Selected 7-alkylidene substituted cephems were synthesized and subjected to antitumor assay. The effect of substituents was examined to establish structure–activity relationships. It was found that the intensive intracellular generation of nitric oxide induced by *tert*-butyl 7-alkylidene cephalosporanate sulfones could be also regarded as an additional cytotoxic factor taking place both in vitro and in vivo experiments. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Recently there were published data describing antimetastatic and antitumor properties both in vitro and in vivo for cepham and oxapenam structural analogues.[1,2] We have also found that some 3-acyloxymethyl substituted *tert*-butyl cephalosporanate sulfones, in addition to inhibiting elastase, unexpectedly behaved as cytotoxic agents against various tumor cell lines and as the inductors of the extensive intracellular NO generation.[3] Similar effects were observed for 6α-chloro and 6,6-dihydro 2β-methyl penicillanate sulfones.[4] The structural similarity of the mentioned compounds with already known elastase and β-lactamase inhibitors[5,6] encouraged us to choose 7-alkylidene substituted cephalosporanate sulfones (already known as potent β-lactamase inhibitors[7]) as a suitable object for the search of new antitumor agents.

Chemistry

The Wittig condensation previously used for the introduction of 7-alkoxycarbonylmethylene, 7-arylmethylene and 7-hetarylmethylene side chain in cephem nucleus [7] was also found to be effective in the preparation of the isomeric mixtures of substances **4a**–**k**. *tert*-Butyl 7-oxocephalosporanate (**2**) which was employed in this reaction was successfully obtained from **1** by two alternative Hagiwara and Buynak procedures.[8,9]

Cephem sulfones **5a**, **5b**, **5e**–**h** prepared by the oxidation of the appropriate cephems **4a**, **4b**, **4e**–**h** were isolated from the reaction mixture as individual 7(Z) and 7(E) isomers using column chromatography. In the case of **5c**, **5i** and **5k**, such fractionation failed and these compounds were obtained as 7(Z) and 7(E) isomer mixtures. Condensation of **2** with acetylphosphorane **3** (R=MeCO) resulted in the formation of only one 7(Z) cephem **4d** which was converted in **5d** with the same geometry of side chain. It was found that the nature of bases used for the conversion of arylphosphonium bromides into ylides **3** (R=Ph, 4-$O_2NC_6H_4$) and their in situ condensation with **2** affected the stereochemistry of the appropriate cephems. Sodium hydride promoted the excessive formation of 7(Z)-isomers **4e**, **4g** and potassium carbonate-7(E)-ones **4f**, **4h**.

The carbonyl group in the side chain of 7(Z)-acetylmethylene cephalosporanate **5d** was subjected to the condensation with alkoxycarbonyl phosphoranes **3** (R=MeOCO, *t*-BuOCO) producing isomeric cephalosporanates **6a**–**c** with 4-alkoxycarbonylbuten-3-ylidene side chain skeleton. Their chromatographic separation was successful only for **6b** and **6c**. Attempts to use arylidene phosporanes **3** (R=Ph, 4-$O_2NC_6H_4$) for such transformation failed.

Deprotection of carboxyl in some cephem esters with trifluoroacetic acid led to the formation of new cephalosporanic acids **7a**–**c**, **8** and already known ones **9**, **10**.[10]

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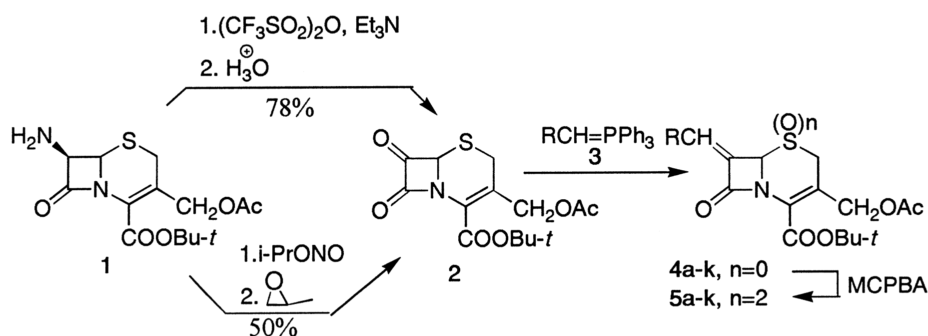
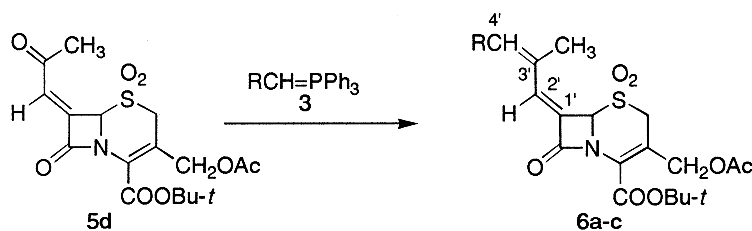


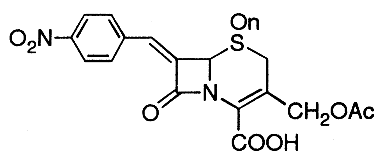
R = t-BuOCO, MeOCO, MeCO, Ph, 4-O$_2$N-C$_6$H$_4$, 4-Pyridyl, 2-Furyl

4, 5	R	Geometry at C7 (Z/E ratio)
4g,4h	4-O$_2$N-C$_6$H$_4$	Z/E (80:20)
5a	*t*-BuOCO	Z (100:0)
5b	*t*-BuOCO	E (0:100)
5c	MeOCO	Z/E (80:20)
5d	MeCO	Z (100:0)
5e	Ph	Z (100:0)
5f	Ph	E (0:100)
5g	4-O$_2$N-C$_6$H$_4$	Z (100:0)
5h	4-O$_2$N-C$_6$H$_4$	E (0:100)
5i	4-Pyridyl	Z/E (80:20)
5k	2-Furyl	Z/E (75:25)

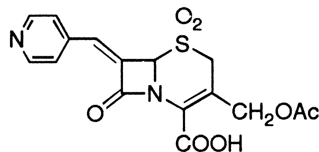


R = MeOCO, t-BuOCO

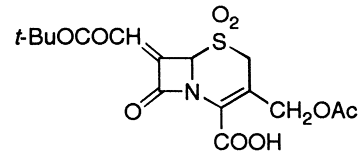
6a-c	R	Side chain geometry (% ratio)
6a	Me	1’Z, 3’Z/ 1Z, 3’E (50:50)
6b	*t*-Bu	1’Z, 3’Z
6c	*t*-Bu	1’Z,3’E



7a n=0 7Z/7E (95:5)
7b n=2 7Z/7E (100:0)
7c n=2 7Z/7E (0:100)



8 Z/E (80:20)



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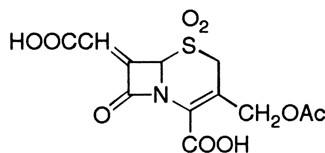

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Biology

Cytotoxic activity of the synthesized compounds in vitro was tested on standard monolayer tumor cell lines: MG-22A (mouse hepatoma), HT-1080 (human fibrosarcoma), B16 (mouse melanoma), Neuro 2A (mouse neuroblastoma) and normal mouse fibroblast cells. Concentrations providing 50% of tumor death effect (TD_{50}) were determined according to the standard procedure using 96-well plates and two independent colorimetric methods:

a. coloration with crystal violet (CV), specifying the integrity of cell membranes;
b. coloration with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), characterizing the redox activity of mitochondrial enzymes in cells.[11,12]

Concentration of NO in supernatant generated by surviving cells in the presence of tested compounds was determined using Greyss' method.[11] Obtained values extrapolated for 100% live cells gave total generation ability parameter TG_{100} which was calculated according to the equation:

$$TG_{100} = G_{EX} \cdot 100/C\,(nM \cdot 10^{-1}/200\,\mu L)$$

where:

G_{EX} = concentration of NO (nM) in supernatant generated by surviving cells in 200 μL volume of plate cell after incubation with maximal tolerable concentration of a test compound (1–100 μg/mL);

C = percentage of the surviving cells after incubation with maximal tolerable concentration of a test compound (CV, coloration).

In vivo curing effect was investigated against melanoma (B16) and sarcoma (S-180) tumors inoculated subcutaneously in mice.

The IC_{50} values characterizing inhibiting properties of cephalosporanic acids and their esters towards *Enterobacter Cloacae* penicillinase and Porcine Pancreas elastase were determined using accordingly cephalotin and standard tetrapeptide *para*-nitroanilide as substrates.

Results and Discussion

The results of cytotoxic experiments in vitro and NO generating ability for the synthesized compounds are summarized in Table 1. The intensity of both effects depended on the nature and stereochemistry of the substituent in the position 7, on the existence of ester protecting group and on the degree of sulfur oxidation.

tert-Butyl cephalosporanate sulfone **5a** with bulky 7(Z)-*tert*-butoxycarbonylmethylene side chain was characterized by the highest cytotoxicity against all tumor cell lines and by the least cytotoxicity towards normal fibroblast cells. Its 7(E)-isomer **5b** exhibited at least 10 times weaker activity against all tumor cells. No such significant difference was observed in activity for other couples of isomeric cephem esters with arylmethylene

Table 1. In vitro cell cytotoxicity and the ability of intracellular NO generation caused by 7-alkylidene cephalosporanate sulfones

Compound	Cell lines													
	MG-22A			HT-1080			B16			Neuro 2A			Fibroblasts	
	TD_{50} (CV)[a]	TD_{50} (MTT)[b]	TG_{100}[c]	TD_{50} (CV)	TD_{50} (MTT)	TG_{100}	TD_{50} (CV)	TD_{50} (MTT)	TG_{100}	TD_{50} (CV)	TD_{50} (MTT)	TG_{100}	TD_{50}	TG_{100}
4g, **4h**	2.2	3.0	300	7.4	6.2	250	2.4	4.5	150	8.0	6.4	150		
5a	0.37	0.73	700	0. 17	0.31	1100	0.10	0.11	850	0.25	0.22	750	4.7	n.t.[f]
5b	22	32	482	4	3	750	7.3	10	210	42	41	600	7	400
5c	48	52	620	46	53	750	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		
5d	55	47	597	83	100	29	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		
5e	4.5	5.2	375	6.8	10	154	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		
5f	2.7	4	451	3	4	978	4	6	750	6	8	750	4.7	38
5g	1.7	0.8	1000	< 1	< 1	1000	< 1	< 1	129	3.0	0.9	200	1.7	
5h	4.8	4.9	900	0.8	1.0	1250	0.3	0.7	189	< 1	5.3	214	4	
5i	4	5	800	2.5	6	650	4	5	622	5	6	800	3.5	
5k	9.8	8.4	650	3.2	1	1000	5.6	6.2	155	3.3	3	340	**[g]	
6b	4.5	6.0	250	3.2	4.1	350	6.2	5.3	250	3.7	4.2	300	6.1	129
6c	3.0	5.0	200	0.8	3.3	350	1.7	2.3	200	4.2	5.0	200	4.6	
7a	1.5	1.4	133	7	6	133	7	5	200	0.3	0.2	150		
7b	0.3	0.3	200	< 1	< 1	150	0.7	0.4	100[d]	0.3	0.3	100[d]	0.8	350
7c	1.5	3.0	200	1.0	0.8	150	0.9	1.4	75[e]	0.3	0.3	33[e]	7	175
8	77	92	15	55	48	300	89	> 100	8	> 100	> 100	48		
10	9	9.7	250	5.9	5.0	250	> 10	> 10	250	> 10	> 10	9	**[g]	7

[a]Concentration (μg/mL) providing 50% cell killing effect (CV, coloration).
[b]Concentration (μg/mL) providing 50% cell killing effect (MTT-coloration).
[c]Extrapolated total NO generation ability at 100 μg/mL concentration of tested compound.
[d]Extrapolated total NO generation ability at 10 μg/mL concentration of tested compound.
[e]Extrapolated total NO generation ability at 1 μg/mL concentration of tested compound.
[f]n.t.- not tested.
[g]The stimulation of cell growth.

and alkoxy-3-butenylidene side chains **5e**, **5f**, **5g**, **5h**, **6b**, **6c**. Their TD_{50} values varied in the range of 1–7 μg/mL for different cell lines but not so unequivocally in the favor of one isomer as in the previous example. Less bulky 7(Z)-acetylmethylene and 7-methoxycarbonylmethylene side chains in **5d** and **5c** caused significant reduction of cytotoxic properties. Cephem sulfone esters **5i** and **5k** representing mixtures of Z and E isomers with 4-pyridylmethylene and 2-furfurylidene side chains exhibited similar level of cytotoxicity against tumor cells and just opposite in the case of fibroblast cells.

It was found that cephems with free carboxyl **7a–c**, **10** also demonstrated potent cytotoxic effect in vitro. In the case of compound **10** there was also observed good selectivity in these properties among tumor and fibroblast cells.

NO generation ability parameter (TG_{100}) for the majority of the cephem esters **5a–k** correlated with the appropriate TD_{50} values. More toxic ones during incubation generated higher NO concentrations in supernatant and vice versa. Cephems with deprotected carboxyl or unoxidized sulfur demonstrated significant reduction of these properties. 7-Nitrobenzylidene substituted cephalosporanates: **4g** and **4h** mixture, **5g**, **5h**, **7a–c** with above mentioned structural differences served as a good example for such tendencies.

On the base of cytotoxic data in vitro compound **5a** has been chosen for the treatment of relatively slowly growing Melanoma (B16) and **5f**-for rapidly growing Sarcoma (S-180) (see Table 2). It was found that the intervals in the administration for 3 and 2 days allowed considerably to diminish toxic side effects complicating the treatment of mice and to obtain tumor growth inhibition exceeding 70% during first 11 days. Cephalosporanic acids **7b** and **7c** with decreased ability of NO generation were much more weaker anticancer agents.

7-Alkylidene substituted cephem esters **4k**, **5a**, **5f**, **5i**, **5k**, **6b** at 1 mM concentration demonstrated the absence of Porcine Pancreas elastase inhibiting properties. Cephalosporanic acids **7c**, **8** at 1 mM concentration inhibited cephalotin hydrolysis catalyzed by Enterobacter Cloacae penicillinase accordingly for 60 and 50%.

Conclusions

Obtained data support the potent antitumor properties of 7-alkylidene substituted cephems. Specific structural features of tested β-lactams do not allow them to mimic cytotoxic compounds with alkylating, intercalating, antimetabolic and other already known types of action mechanism. However, their structural resemblance with β-lactamase as well as with elastase inhibitors confirms the possibility of similar behavior against specific proteases responsible for the metastasis and proliferation of tumor cells.[13] The excessive intracellular generation of nitric oxide responsible for the damage of enzymes and DNA could be also regarded as an additional cytotoxic factor taking place both in vitro and in vivo experiments.[14]

Experimental

General

Melting points were determined on a Boetius PHMK 05 instrument and uncorrected. Proton magnetic resonance spectra were recorded on a Bruker WH-90 spectrometer (90 MHz). All chemical shifts were registered as δ values (ppm) in regard to the internal tetramethylsilane and *J* (coupling constant) in Hz. IR spectra were obtained on a Perkin–Elmer 580B spectrophotometer. Microanalytical data were obtained on a Carlo Erba 1108 apparatus; they were performed for C, H, N and the results were within 0.4% of theoretical values. HPLC analyses were performed on a Dupont Model 8800 chromatograph with UV detector ($\lambda = 254$ nm). All reactions were monitored by TLC carried out on Merck Kieselgel plates using UV light as a visualizing agent. Merck Kieselgel (0.063–0.230 mm) was used for preparative column chromatography. Optical density in biological tests was measured with a horizontal spectrophotometer Tetertek Multiscan MCC/340. The commercially available (Aldrich, Acros and Sigma) reagents were employed in this study.

Synthetic procedures

***tert*-Butyl 7-oxocephalosporanate (2).** The title compound was prepared according to Hagiwara[8] (90%) or

Table 2. Therapeutic effect of 7-alkylidenecephalosporanates on the treatment of tumour in mice

Compound	Tumour type	Administration schedule, days	Dosage, mg/kg/day	Tumor growth inhibition GI%			
				In 7 days	In 9 days	In 11 days	In 16 days
5a	Melanoma (B16)	1, 2, 3... 7, 8, 9...11...14, 15	3		69 $P = 0.180$	77 $P = 0.228$	24 $P = 0.491$
5f	Sarcoma (S-180)	1, 2, 3... 7, 8, 9...11...14, 15, 16	3	51 $P = 0.123$	75 $P = 0.128$	76 $P = 0.093$	*[a]
7b	Sarcoma (S-180)	1, 2, 4, 7	2	18 $P = 0.345$	14 $P = 0.214$		
7c	Sarcoma (S-180)	1, 2, 4, 7	2	10 $P = 0.126$	10 $P = 0.299$		

[a]100% survival of treated mice versus to control group, tumor volume remained the same as in the previous measurement.

Buynak procedure [9] (50%) as a brown solid which was used in following reactions without purification, R_f = 0.5, EtOAc:hexane, 1:1. A sample purified by column chromatography (EtOAc:hexane, 1:1) was characterized by following spectroscopic data. ^{1}H NMR ($CDCl_3$) δ: 1.55 (s, 9H, C_4H_9); 2.09 (s, 3H, CH_3); 3.35, 3.66 (AB-q, J = 18 Hz, 2H, SCH_2); 4.82, 5.13 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.29 (s, 1H, 6-H). IR (nujol) 1820, 1785, 1745, 1720, 1640 cm^{-1}.

***tert*-Butyl 7-(4-nitrobenzylidene)cephalosporanate sulfone 7Z and 7E isomers mixture (4g, 4h).** NaH (122 mg, 3.06 mmol 60% suspension in oil) and 15-crown-5 (10 mg) were added to a suspension of 4-nitrophenyltriphenylphosphonium bromide (1.33 g, 3.06 mmol) and **2** (1.00 g, 3.06 mmol) in dry CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 4 h, washed with 3% HCl, dried over (Na_2SO_4), concentrated and the residue was purified by column chromatography using EtOAc:hexane, 1:2 to give 0.40 g (59%) of *tert*-butyl 7-(4-nitrobenzylidene)cephalosporanate as a thick oil, consisting from Z and E isomers (80:10) and 93% purity according to HPLC (Nova Pak Silicon, EtOAc:hexane, 12:88 as mobile phase). TLC R_f = 0.45 (EtOAc:hexane, 1:2). ^{1}H NMR for Z isomer ($CDCl_3$, TMS) δ: 1.51 (s, 9H, C_4H_9); 2.02 (s, 3H, CH_3); 3.31, 3.57 (AB-q, J = 18 Hz, 2H, SCH_2); 4.71, 5.00 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.41 (d, J = 1 Hz, 1H, C_6-H); 7.11 (d, J = 1, 1H, Ph-CH=); 7.55, 8.20 (d, d, J = 9 Hz, 4H, C_6H_4). ^{1}H NMR for E isomer ($CDCl_3$, TMS) δ: 1.53 (s, 9H, C_4H_9); 2.02 (s, 3H, CH_3); 3.28, 3.55 (AB-q, J = 18 Hz, 2H, SCH_2); 4.71, 5.00 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.16 (d, J = 1 Hz, 1H, C_6-H); 6.66 (d, J = 1 Hz, 1H, Ph-CH=); 8.02, 8.22 (d, d, J = 9 Hz, 4H, C_6H_4).

***tert*-Butyl 7-[(Z)-(*tert*-butoxycarbonyl)methylene]cephalosporanate sulfone (5a).** The powder of [(*tert*-butoxycarbonyl)methylene]triphenylphosphorane (**3**) (R = *t*-BuOCO, 1.96 g, 5.22 mmol) was added to a solution of *tert*-butyl 7-oxocephalosporanate (**2**) (1.7 g, 5.22 mmol) in dry CH_2Cl_2 (15 mL). The mixture was stirred at room temperature for 2 h, concentrated and the residue was purified by column chromatography using EtOAc: hexane, 1:3 to give 0.7 g (52%) of *tert*-butyl 7-[(*tert*-butoxycarbonyl)methylene]cephalosporanate as a mixture of Z and E isomers. It was dissolved in CH_2Cl_2 (20 mL) and at 0 °C was treated with *m*-CPBA (85%, 424 mg, 2.09 mmol). The mixture was stirred at 0 °C for 2 h and at room temperature for 1.5 h. The organic phase was washed with saturated $NaHCO_3$, brine, dried over (Na_2SO_4) and concentrated. Column chromatography (EtOAc: hexane, 1:2) afforded 120 mg (37%) of the title product **5a**: mp 50 °C. TLC R_f = 0.60 (EtOAc:hexane, 1:2). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.54 (s, 9H, C_4H_9); 1.56 (9H, s, C_4H_9); 2.13 (s, 3H, CH_3); 3.71, 4,08 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.80, 5.20 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.53 (br.s, 1H, 6-H); 6.55 (d, J = 0.8 Hz, 1H, -CH=). Anal. calcd for $C_{20}H_{27}NO_9S$ (0.25 C_6H_{14}): C 53.91; H 6.41; N 2.92. Found: C 53.69; H 6.28; N 2.90.

***tert*-Butyl 7-[(E)-(*tert*-butoxycarbonyl)methylene]cephalosporanate sulfone (5b).** This substance was isolated from the reaction mixture obtained during the preparation of compound **5a** yielding 60 mg (18%) of **5b** as foam, 94% purity according to HPLC (μ Porasil, EtOAc: hexane, 20:80 as mobile phase). TLC R_f = 0.25 (EtOAc: hexane, 1:2). ^{1}H NMR spectra: ($CDCl_3$, TMS) δ: 1.51 (s, 18H, 2C_4H_9); 2.08 (s, 3H, CH_3); 3.71, 4,04 (AB-q, J = 19 Hz, 2H, SO_2CH_2);); 4.71, 5.08 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.17 (br.s, 1H, 6-H); 6.24 (d, J = 0.8 Hz, 1H, -CH=).

***tert*-Butyl 7-[(methoxycarbonyl)methylene]cephalosporanate sulfone (5c).** Prepared from **2** and **3** (R = MeOCO) as described for **5a** to give 47% of *tert*-butyl 7-[(methoxycarbonyl)methylene]cephalosporanate as a mixture of Z and E isomers (EtOAc:petroleum ether, 1:1). Its oxidation by *m*-CPBA and purification by column chromatography (EtOAc:hexane, 1:2) afforded title product **5c** as a mixture of Z and E isomers (80:20) in 48% yield. TLC R_f = 0.20 (EtOAc:hexane, 1:2). ^{1}H NMR spectra for Z isomer ($CDCl_3$, TMS) δ: 1.55 (s, 9H, C_4H_9); 2.11 (s, 3H, CH_3); 3.75, 4,11 (AB-q, J = 19 Hz, 2H, SO_2CH_2); 3.84 (s, 3H, CH_3O); 4.77, 5.13 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.55 (br.s, 1H, 6-H); 6.64 (d, J = 0.5 Hz, 1H,-CH=). ^{1}H NMR spectra for E isomer ($CDCl_3$, TMS) δ: 1.55 (s, 9H, C_4H_9); 2.11 (s, 3H, CH_3); 3.71, 4,04 (AB-q, J = 19 Hz, 2H, SO_2CH_2); 3.84 (s, 3H, CH_3O); 4.71, 5.11 (AB-q, J = 14 Hz, 2H, CH_2 OCO); 5.55 (br.s., 1H, 6-H); 6.31 (d, J = 0.5 Hz, 1H, -CH=). Anal. calcd for $C_{17}H_{27}NO_9S$(0.25 H_2O): C 48.62; H 5.15; N 3.33. Found: C 48.47; H 5.07; N 3.29.

***tert*-Butyl 7-[(Z)-(acetyl)methylene]cephalosporanate sulfone (5d).** Prepared from **2** and **3** (R = MeCO) as described for **5a** to give 71% *tert*-butyl 7-[(Z)-(acetyl)-methylene]cephalosporanate (EtOAc:petroleum ether, 1:1). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.60 (s, 9H, C_4H_9); 2.11 (s, 3H, CH_3); 2.40 (s, 3H, CH_3COC=); 3.33, 3.66 (AB-q, J = 19 Hz, 2H, SO_2CH_2); 4.80, 5.06 (AB-q, J = 12 Hz, 2H, CH_2OCO); 5.46 (d, J = 0.5 Hz, 1H, 6-H); 6.71 (d, J = 0.5 Hz, 1H,-CH=). Its oxidation with *m*-CPBA and purification by column chromatography (EtOAc:hexane, 1:2) afforded title product **5d** in 58% yield: mp 133–135 °C. TLC R_f = 0.20 (EtOAc:hexane, 1:2). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.60 (s, 9H, C_4H_9); 2.11 (s, 3H, CH_3); 2.44 (s, 3H, CH_3COC=); 3.75, 4.13 (AB-q, J = 19 Hz, 2H, SO_2CH_2); 4.77, 5.15 (AB-q, J = 12 Hz, 2H, CH_2OCO); 5.62 (br.s, 1H, 6-H); 6.71 (d, J = 0.5 Hz, 1H,-CH=). Anal. calcd for $C_{17}H_{21}NO_8S$: C 51.12; H 5.30; N 3.51. Found: C 50.78; H 5.26; N 3.61.

***tert*-Butyl 7-[(Z)-benzylidene]cephalosporanate sulfone (5e).** To a suspension of benzyltriphenylphosphonium bromide (1.33 g, 3.06 mmol) and **2** (1.00 g, 3.06 mmol) in dry CH_2Cl_2 (20 mL) was added NaH (285 mg, 7.12 mmol, 60% dispersion in mineral oil) and 15-crown-5 (10 mg). The mixture was stirred at room temperature for 2 h, washed with 3% HCl, dried (Na_2SO_4), concentrated and the residue was purified by column chromatography using EtOAc:hexane, 1:2 to give 0.30 g (24%) of *tert*-butyl 7-(benzylidene)cephalosporanate as a mixture of Z and E isomers. It was dissolved in CH_2Cl_2 (20 mL) and treated with *m*-CPBA (85%, 609 mg, 3.00 mmol) at 0 °C. The mixture was stirred at 0 °C for 1 h and at room temperature for 3 h, washed with

saturated $NaHCO_3$, brine, dried over (Na_2SO_4) and concentrated. Column chromatography (EtOAc:hexane, 1:2) afforded 87 mg (27%) of the title product **5e**: mp 147–149 °C, 94% purity according to HPLC (Nova Pak Silicon, EtOAc:hexane, 20:80 as mobile phase). TLC R_f = 0.23 (EtOAc:hexane, 1:2). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.60 (s, 9H, C_4H_9); 2.09 (s, 3H, CH_3); 3.78, 4.11 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.75, 5.17 (AB-q, J = 13 Hz, 2H, CH_2OCO); 5.62 (br.s, 1H, 6-H); 7.28 (br.s, 1H, -CH=); 7.37–7.60 (m, 3H, 3-H, 4-H, 5-H in Ph); 7.60–7.82 (m, 2H, 2-H, 6-H in Ph).

***tert*-Butyl 7-[(E)-benzylidene]cephalosporanate sulfone (5f).** K_2CO_3 (422 mg, 3.06 mmol) and 18-crown-6 (10 mg) were added to a suspension of benzyltriphenylphosphonium bromide (1.33 g, 3.06 mmol) and **2** (1.00 g, 3.06 mmol) in dry CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 2 h washed with 3% HCl, dried over (Na_2SO_4), concentrated and the residue was purified by column chromatography using EtOAc: hexane, 1:2 to give 0.30 g (24%) of *tert*-butyl 7-(benzylidene)cephalosporanate as a mixture of Z and E isomers (3:5). It was oxidized as described for **5e** to give 150 mg (45%) of the title product **5f** as foam, 92% purity according to HPLC (Nova Pak Silicon, EtOAc: hexane, 20:80 as mobile phase). TLC R_f = 0.11 (EtOAc: hexane, 1:2). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.51 (s, 9H, C_4H_9); 2.11 (s, 3H, CH_3); 3.71, 4.06 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.73, 5.02 (AB-q, J = 13 Hz, 2H, CH_2OCO); 5.24 (br.s, 1H, 6-H); 6.82 (br.s, 1H, -CH=); 7.33–7.55 (m, 3H, 3-H, 4-H, 5-H in Ph); 7.78–7.95 (m, 2H, 2-H, 6-H in Ph).

***tert*-Butyl 7-[(Z)-4-nitrobenzylidene]cephalosporanate sulfone (5g).** Prepared from **2** and 4-nitro-benzyltriphenylphosphonium bromide as described for **5f** to give 59% of *tert*-butyl 7-(4-nitrobenzylidene)cephalosporanate as a mixture of Z and E isomers (EtOAc:hexane, 1:2). Its oxidation with *m*-CPBA and purification by column chromatography (EtOAc:hexane, 1:2) afforded 130 mg (30%) of the title product **5g**: mp 172–174 °C, TLC R_f = 0.28 (EtOAc:hexane, 1:2). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.58 (s, 9H, C_4H_9); 2.02 (s, 3H, CH_3); 3.80, 4.15 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.75, 5.11 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.62 (d, J = 1 Hz, 1H, 6-H); 7.47 (br.s, 1H, -CH=); 7.86, 8.20 (d, d, J = 9 Hz, 4H, C_6H_4). Anal. calcd for $C_{21}H_{22}N_2O_9S$: C 52.72; H 4.63; N 5.85. Found: C 52.75; H 4.79; N 5.84.

***tert*-Butyl 7-[(E)-4-nitrobenzylidene]cephalosporanate sulfone (5h).** This substance was isolated from reaction mixture obtained during the preparation of compound **5g** yielding 150 mg (35%) of **5h**: mp 162–164 °C, TLC R_f = 0.14 (EtOAc:hexane, 1:2). ^{1}H NMR ($CDCl_3$, TMS) δ: 1.51 (s, 9H, C_4H_9); 2.04 (s, 3H, CH_3); 3.77, 4.11 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.73, 5.15 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.29 (d, J = 1 Hz, 1H, 6-H); 6.95 (br.s, 1H,-CH=); 8.04, 8.17 (d, d, J = 9 Hz, 4H, C_6H_4). Anal. calcd for $C_{21}H_{22}N_2O_9S(0.25C_6H_{14})$: 54.04; H 5.13; N 5.62. Found: C 53.77; H 5.03; N 5.41.

***tert*-Butyl 7-[4-(pyridyl)methylene]cephalosporanate sulfone (5i).** Prepared from **2** and pyridylmethyltriphenylphosphonium bromide (R = 4-Pyridyl) as described for **5f** to give 48% of *tert*-butyl 7-(4-pyridylmethylene)-cephalosporanate as a mixture of Z and E isomers (EtOAc:hexane, 2:1). Its oxidation by *m*-CPBA and purification by column chromatography (EtOH: CH_2Cl_2, 1:2) afforded 300 mg (46%) of the title product **5i** as a mixture of Z and E isomers (80:20): mp 119–123 °C, TLC R_f = 0.77 (EtOH:CH_2Cl_2, 1:2), 92% purity according to HPLC (Supelcosil LC-Si, *iso*-PrOH:hexane, 40:60 as mobile phase). ^{1}H NMR for Z isomer ($CDCl_3$, TMS) δ: 1.51 (s, 9H, C_4H_9); 2.06 (s, 3H, CH_3); 3.16–4.35 (m, 2H, SO_2CH_2); 4.71, 5.09 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.66 (br. s, 1H, 6-H); 7.26 (br.s, 1H,-CH=); 7.57, 8.15 (d, d, J = 6 Hz, 4H, C_6H_4N). ^{1}H NMR for E isomer ($CDCl_3$, TMS) δ: 1.51 (s, 9H, C_4H_9); 2.06 (s, 3H, CH_3); 3.16–4.35 (m, 2H, SO_2CH_2); 4.71, 5.09 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.29 (br. s, 1H, 6-H); 6.73 (br.s, 1H, -CH=); 7.89, 8.15 (d, d, J = 6 Hz, 4H, C_6H_4N).

***tert*-Butyl 7-[2-(furyl)methylene]cephalosporanate sulfone (5k).** Prepared from **2** and furfuryltriphenylphosphonium bromide as described for **5f** to give 25% of *tert*-butyl 7-(2-furylmethylene)cephalosporanate as a mixture of Z and E isomers (EtOAc:hexane 1:2). Its oxidation with *m*-CPBA and purification by column chromatography (EtOAc:hexane, 1:1) afforded 37% of the title product **5k** as a mixture of Z and E isomers (75:25): mp 147–150 °C, TLC R_f = 0.37 (EtOAc:hexane, 1:1), 97% purity according to HPLC (Supelcosil LC-Si, EtOAc:hexane, 50:50 as mobile phase). ^{1}H NMR for Z isomer ($CDCl_3$, TMS) δ: 1.62 (s, 9H, C_4H_9); 2.04 (s, 3H, CH_3); 3.73, 4.06 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.75, 5.13 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.57 (d, J = 1 Hz, 6-H,); 6.55 (m, 1H, 4-H in furan); 6.88 (d, J = 4 Hz, 1H, 3-H in furan); 7.17 (d, J = 1 Hz, 1H,-CH=); 7.60 (m, 5-H in furan). ^{1}H NMR for E isomer ($CDCl_3$, TMS) δ: 1.63 (s, 9H, C_4H_9); 2.04 (s, 3H, CH_3); 3.71, 4.01 (AB-q, J = 18 Hz, 2H, SO_2CH_2); 4.75, 5.13 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.22 (br.s., 6-H,); 6.55 (m, 1H, 4-H in furan); 6.82 (br.s., 1H,-CH=); 7.46 (d, J = 4 Hz, 1H, 3-H in furan); 7.60 (m, 1H, 5-H in furan). Anal. calcd for $C_{19}H_{21}$ NO_8S: C 53.89; H 4.99; N 3.31. Found: C 53.54; H 5.24 ; N 3.13.

***tert*-Butyl 7-[(Z)-4-methoxycarbonyl-2-methyl-3-butenylidene]cephalosporanate sulfone (6a).** The suspension of **3** (R = MeCO, 50 mg, 0.12 mmol) and **5d** (50 mg, 0.12 mmol in dry CH_2Cl_2 (20 mL) was boiled for 2 h and concentrated. Column chromatography (EtOAc:hexane, 1:1) afforded 10 mg (17%) of the title product **6a** as a mixture of 3′-Z and 3′-E isomers (50:50). TLC R_f = 0.37 (EtOAc:hexane, 1:1), 97% purity according to HPLC (Supelcosil LC-Si, EtOAc:hexane, 50:50 as mobile phase). ^{1}H NMR spectra for 3′-Z isomer ($CDCl_3$, TMS) δ: 1.55 (s, 9H, *t*-Bu); 2.11 (s, 3H, $OCOCH_3$); 2.26 (s, 3H, CH_3); 3.76, 4.08 (AB-q, J = 19 Hz, 2H, SO_2CH_2); 3.77 (s, 3H, OCH_3); 4.77, 5.15 (AB-q, J = 14 Hz, 2H, CH_2OCO); 5.48 (br.s, 1H, 6-H); 6.13 (br.s, 1H, 4′-H) 8.44 (br. s, 1H, 2′-H). ^{1}H NMR spectra for 3′-E isomer ($CDCl_3$, TMS) δ: 1.60 (s, 9H, *t*-Bu); 2.11 (s, 3H, $OCOCH_3$); 2.46 (s, 3H, CH_3); 3.75, 4.08 (AB-q, J = 19 Hz, 2H, SO_2CH_2); 3.77 (s, 3H, OCH_3); 4.77, 5.15 (AB-q,

$J = 14$ Hz, 2H, CH_2OCO); 5.53 (br.s, 1H, 6-H); 6.20 (br.s, 1H, 4′-H) 7.04 (br. s, 1H, 2′-H).

***tert*-Butyl 7-[(Z)-4-*t*-butoxycarbonyl-2-methyl-3-(Z)-butenylidene]cephalo-sporanate sulfone (6b).** Prepared from **3** (R = *t*-BuOCO) and **5d** as described for **6a** to give title product **6b** in 25% yield: mp 163–164 °C. TLC $R_f = 0.51$ (EtOAc:hexane, 1:1). ^1H NMR ($CDCl_3$, TMS) δ: 1.51 (s, 9H, 4′-*t*-Bu); 1.57 (s, 9H, *t*-Bu); 2.11 (s, 3H, $OCOCH_3$); 2.17 (d, $J = 0.5$ Hz, CH_3); 3.71, 4.04 (AB-q, $J = 19$ Hz, 2H, SO_2CH_2); 4.71, 5.11 (AB-q, $J = 14$ Hz, 2H, CH_2OCO); 5.44 (br.s, 1H, 6-H); 6.00 (br.s, 1H, 4′-H) 8.44 (br. s, 1H, 2′-H). Anal. calcd for $C_{23}H_{31}NO_9S(0.4C_6H_{14})$: C 57.34; H 6.92; N 2.63. Found: C 57.11; H 6.58; N 2.52.

***tert*-Butyl 7-[(Z)-4-*t*-butoxycarbonyl-2-methyl-3-(E)-butenylidene]cephalosporanate sulfone (6c).** This substance was isolated from reaction mixture during the preparation of compound **6b** yielding 18 mg (14%) of **6c** as foam $R_f = 0.37$ (EtOAc:hexane, 1:1), 94% purity according to HPLC (Supelcosil LC-Si, EtOAc:hexane, 50:50 as mobile phase). ^1H NMR ($CDCl_3$, TMS) δ: 1.53 (s, 9H, 4′-*t*-Bu); 1.60 (s, 9H, *t*-Bu); 2.11 (s, 3H, $OCOCH_3$); 2.44 (d, $J = 0.5$ Hz, 3H, CH_3); 3.75, 4.11 (AB-q, $J = 19$ Hz, 2H, SO_2CH_2); 4.78, 5.15 (AB-q, $J = 14$ Hz, 2H, CH_2OCO); 5.53 (br.s, 1H, 6-H); 6.11 (br.s, 1H, 4′-H) 7.02 (br. s, 1H, 2′-H).

7-[(Z)-4-Nitrobenzylidene]cephalosporanic acid (7a). The solution of **4g** and **4h** mixture (100 mg, 0.22 mmol) in trifluoroacetic acid (2 mL) and dry CH_2Cl_2 (0.5 mL) was stirred at 0 °C for 1 h and 1 h at room temperature, diluted with CH_2Cl_2 (20 mL), washed with saturated NH_4Cl, dried over (Na_2SO_4) and concentrated. Column chromatography (EtOAc:hexane, 1:3) and repeatedly (EtOAc) afforded 68 mg (80%) of the title product **7a** as a mixture of Z and E isomers (95:5): TLC $R_f = 0.68$ (EtOAc), 93% purity according to HPLC (Zorbax SB-C-18, 0.1 M pH 2.5 phosphate buffer and acetonitrile 40:60 mixture as mobile phase). ^1H NMR for Z isomer (DMSO-d_6, TMS) δ: 2.15 (s, 3H, CH_3); 3.48, 3.84 (AB-q, $J = 18$ Hz, 2H, SCH_2); 4.98 (s, 2H, CH_2OCO); 5.57 (br.s, 1H, 6-H); 7.33 (br.s, 1H, Ph-CH=); 7.71, 8.28 (d, d, $J = 8$ Hz, 4H, C_6H_4).

7-[(Z)-4-Nitrobenzylidene]cephalosporanic acid sulfone (7b). Prepared from **5g** as described for **7a** affording the title product **7g** in 90% yield: mp 191–192 °C, TLC $R_f = 0.23$ (EtOAc:hexane, 1:1), 96% purity according to HPLC (Symmetry C_{18}, 0.1 M pH 2.5 phosphate buffer and acetonitrile 70:30 mixture as mobile phase). ^1H NMR (DMSO-d_6, TMS) δ: 2.00 (s, 3H, CH_3); 4.00, 4.40 (AB-q, $J = 18$ Hz, 2H, SO_2CH_2); 4.60 (s, 2H, CH_2OCO); 6.49 (br.s, 1H, 6-H); 7.15 (s, 1H, COOH); 7.71 (br.s, 1H,-CH=); 8.00, 8.26 (d, d, $J = 9$ Hz, 4H, C_6H_4).

7-[(E)-4-Nitrobenzylidene]cephalosporanic acid sulfone (7c). Prepared from **5h** as described for **6a** affording the title product **7c** in 90% yield: mp 115–117 °C. TLC $R_f = 0.23$ (EtOAc:hexane, 1:1) 92% purity according to HPLC (Symmetry C_{18}, 0.1 M pH 2.5 phosphate buffer and acetonitrile 70:30 mixture as mobile phase). ^1H NMR (DMSO-d_6, TMS) δ: 2.00 (s, 3H, CH_3); 4.00, 4.40 (AB-q, $J = 18$ Hz, 2H, SO_2CH_2); 4.60 (s, 2H, CH_2OCO); 5.93 (br.s, 1H, 6-H); 7.17 (s, 1H, COOH); 7.28 (br.s, 1H, -CH=); 8.33 (s, 4H, C_6H_4).

7-[4-(Pyridyl)methylene]cephalosporanic acid sulfone (8). Prepared from **5i** as described for **7a** affording the title product **7i** in 80% yield as a mixture of Z and E isomers (80:20): TLC $R_f = 0.23$ (EtOAc:hexane 1:1), 98% purity according to HPLC (Supelcosil LC-Si, EtOAc:hexane, 50:50 as mobile phase). ^1H NMR for Z isomer (DMSO-d_6, TMS) δ: 2.04 (s, 3H, CH_3); 4.37 (s, 2H, CH_2OCO); 4.62, 5.04 (AB-q, $J = 13$ Hz, 2H, SO_2CH_2); 6.51 (br.s, 1H, 6-H); 7.60 (br.s, 1H,-CH=); 7.73, 8.31 (d, d, $J = 7$ Hz, 4H, C_5H_4N). ^1H NMR for E isomer (DMSO-d_6, TMS) δ: 2.04 (s, 3H, CH_3); 4.34 (s, 2H, CH_2OCO); 4.62, 5.06 (AB-q, $J = 13$ Hz, 2H, SO_2CH_2); 6.97 (br.s, 1H, 6-H); 7.17 (br.s, 1H,-CH=); 8.00, 8.31 (d, d, $J = 7$ Hz, 4H, C_5H_4N).

7-[(Z)-(*tert*-Butoxycarbonyl)methylene]cephalosporanic acid sulfone (9). Prepared from **5a** as described for **7a** affording the title product **9** in 7% yield: mp 115–117 °C (dec.) which was identical to that published in Buynak's work.[7] TLC $R_f = 0.17$ (CH_2Cl_2:MeOH, 2:1). ^1H NMR ($CDCl_3$, TMS) δ: 1.60 (s, 9H, C_4H_9); 2.15 (s, 3H, CH_3); 3.80, 4,15 (AB-q, $J = 19$ Hz, 2H, SO_2CH_2); 4.77, 5.15 (AB-q, $J = 14$ Hz, 2H, CH_2OCO); 5.66 (br.s, 1H, 6-H); 6.62 (d, $J = 0.5$ Hz, 1H,-CH=); 7.00 (s, 1H, COOH).

7-[(Z)-Carboxymethylene]cephalosporanic acid sulfone (10). This substance was isolated from the reaction mixture obtained during the preparation of compound **9** yielding 90 mg (81%) of **10**, TLC $R_f = 0.00$ (CH_2Cl_2:MeOH, 2:1) which was identical to that published in Buynak's work.[10] ^1H NMR (DMSO-d_6, TMS) δ: 2.04 (s, 3H, CH_3); 4.17, 4,44 (AB-q, $J = 12$ Hz, 2H, SO_2CH_2); 4.62, 5.06 (AB-q, $J = 14$ Hz, 2H, CH_2OCO); 6.22 (br.s, 1H, 6-H); 6.71 (d, $J = 0.5$ Hz, 1H,-CH=); 11.0 (s, 2H, 2COOH).

Biological methods

In vitro cytotoxicity assay and nitric oxide generation in cells. Monolayer cells were cultivated for 72 h in DMEM standard medium without an indicator and antibiotics. After the ampoule was defreezed not more than four passages were performed. The control cells and cells with tested substances in the range of $2–5 \times 10^4$ cell/mL concentration (depending on line nature) were placed on a separate 96 wells plates. Solutions containing the test compounds were diluted and added in wells to give the final concentrations in the range of 100–0.01 μg/mL. Control cells were treated in the same manner only in the absence of test compounds. Plates were cultivated for 72 h. A quantity of survived cells was determined using crystal violet (CV) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) coloration which was assayed by multiscan spectrophotometer. The quantity of alive cells on control plate was taken in calculations for 100%. Concentration of NO in plate wells was determined according to ref 11 after incubation with the tested compounds for 72 h.

Antitumor effect on mice. Melanoma (B16) 1×10^6 cells were inoculated subcutaneously to male $C_{57}BL_6$/DBA2F1 mice (6 weeks old) on day 0. The same procedure was performed for sarcoma (S-180) which in the dosage 5×10^6 cells was inoculated to male ICR mice (6 weeks old) on day 0. Tested compounds were administered ip in the form of 2% DMSO solutions containing 0.3% agarose additive on days 1, 2, 3, 7, 8, 9, 11, 14, 15,16. In the case of S-180 the experiments were ended on the 9th day. The treatment with **5f** lasted 16 days due to good condition of animals. The number of mice in the treated group varied from 3 to 6. The number of mice in the control group (K) was determined from equation:

$$K = O^{1/2} \cdot M$$

where:

O = the number of the treated groups,
M = the number of mice in every group.

The value of tumour growth inhibition (GI%) was estimated by comparing tumour volume in the treated and control mice groups from the following equation:

$$GI\% = (1.00 - E/V)\,100$$

where

E = tumour volume in the treated group
V = tumour volume in the control group

The tumour volume (V) was calculated from equation:

$$V = 4\pi AB^2/3$$

where A and B are ellipsoid maximum and minimum diameters (calculated volume was reduced by 2 times in the case of flat tumour shape).

Inhibition of porcine pancreas elastase. The inhibiting effect for the tested compounds was determined according to a standard colorimetric procedure adapted on 96 wells plates based on the measurement of Porcine Pancreatic elastase (Type III) amidolytic activity using a standard tetrapeptide *para*-nitroanilide as substrate.[3]

Inhibition of β-lactamase. The inhibition of cephalotin hydrolysis catalyzed by Enterobacter Cloacae penicillinase was tested according to standard procedure.[15]

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