Eur. J. Med. Chem. 33 (1998) 755–762

New biological properties of *tert*-butyl cephalosporanate sulfones [1]

Grigory Veinberg*, Irina Shestakova, Nora Grigan, Dan Musel, Iveta Kanepe, Ilona Domrachova, Vera Grigoryeva, Olga Zharkova, Ivars Turovskis, Ivars Kalvinsh, Andris Strakovs, Edmunds Lukevics

Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV 1006, Latvia

(Received 20 November 1997; accepted 29 April 1998)

Abstract – *tert*-Butyl cephalosporanate 1,1-dioxides variously substituted in positions 7 and 3 were obtained from 7-amino-deacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA). It was found that the cephalosporins containing Aspirin and Diclofenac in a prodrug form in their 3-acyloxymethyl moiety release them after hydrolytic splitting of the β-lactam ring. They also demonstrated high efficacy as elastase inhibitors. Two of them stimulated the biosynthesis of nitric oxide in RAW 264.7 macrophages cells. The same effect observed in tumor and normal cells in the presence of cephalosporins was accompanied by cytotoxic effect in vitro. © Elsevier, Paris

***tert*-butyl cephalosporanate 1,1-dioxides / Aspirin / Diclofenac / basic hydrolysis / elastase inhibition / antiinflammatory and cytotoxic properties / nitric oxide generation**

1. Introduction

It is known that the modification of the structure and configuration of substituents in β-lactam antibiotics permits to synthesize selective inhibitors of different enzymes acting not only in the bacterial but also in the mammalian cells. Suppression of the catalytic properties of these enzymes leads to the manifestation of the appropriate therapeutic effects:

(a) antibacterial activity in the case of transpeptidase inhibition [1];

(b) protection of the first and second generation of penicillins and cephalosporins from the inactivation by β-lactamases produced in resistant strains of bacteria using as inhibitors derivatives of these antibiotics or clavulanic acid [2];

(c) prevention of Elastase-mediated connective-tissue destruction in the case of Elastase inhibition [3];

(d) antitumor properties of oxapenams both in vitro and in vivo on the basis of still unknown mechanism [4].

On these grounds we undertook the synthesis and biological evaluation of new *tert*-butyl cephalosporanate 1,1-dioxides including ones containing the prodrug forms of Aspirin or Diclofenac at the position C-3. Liberation of the latters occurring synchronously with the splitting of the β-lactam ring participating in Elastase acylation should trigger inhibition of cyclooxygenase [5] and display antiinflammatory effects.

2. Chemistry

Already existing and specially devised chemical methods were used for the preparation of the targeted Dual Action Cephalosporins (DAC): **5–8** by means of two alternative pathways: reactions (a) and (b) (*figure 1*) [3, 6–9]. The first one starting from 7-ADCA included its esterification with *tert*-butyl acetate in the presence of boron trifluoride etherate, sulfur oxidation with hydrogen peroxide, diazotation of amino group with *iso*-propyl

*Correspondence and reprints

[1]Preliminary results were presented at the 5th Conference of Chemical Synthesis of Antibiotics and Related Microbial Products, 1996, in Debrecen, Hungary, and in the paper published in Bioorg. Med. Chem. Lett. 7 (1997) 843–846.

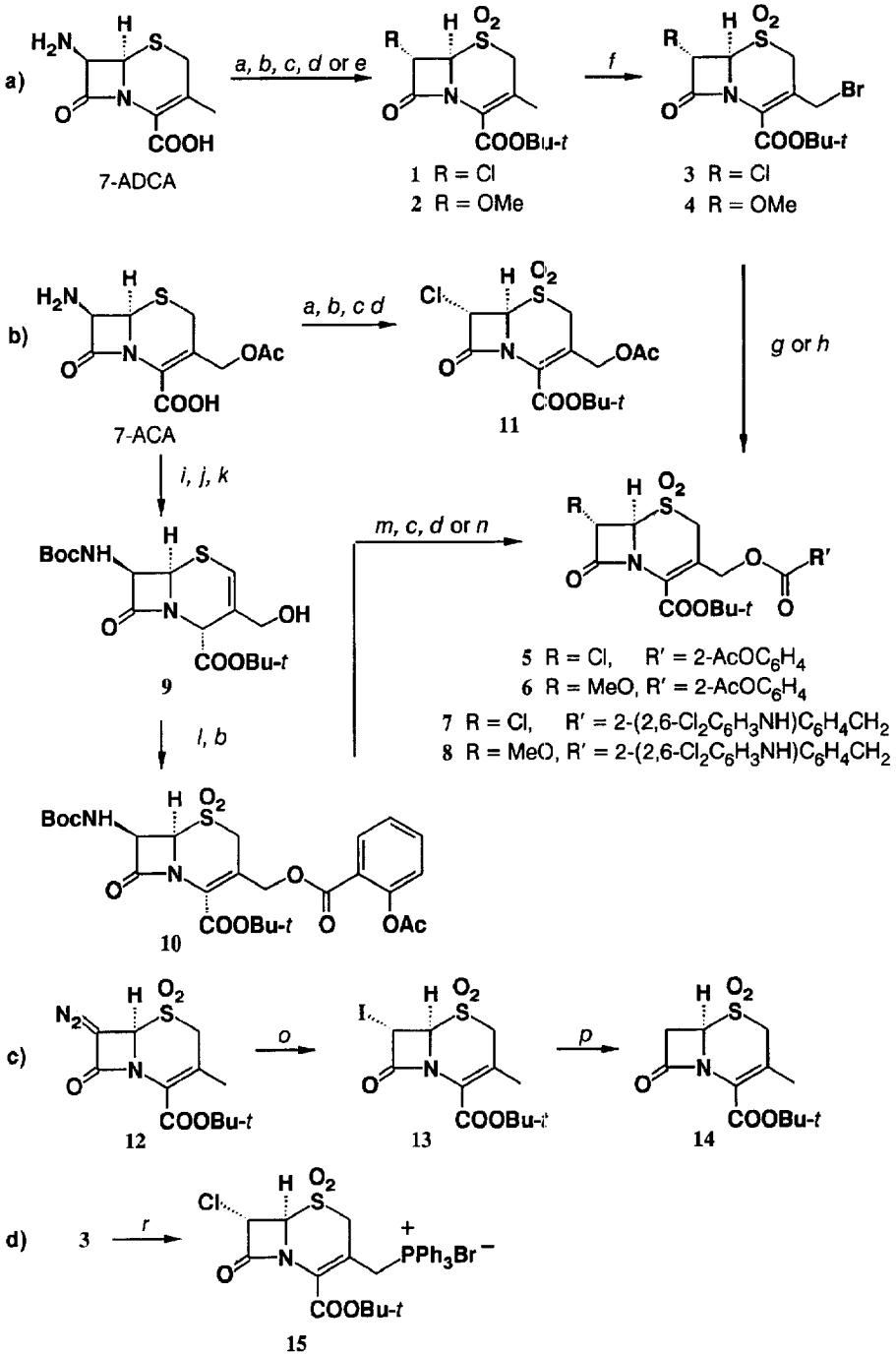

Figure 1. Reagents: *a*: *t*-BuOAc, $BF_3 \bullet Et_2O$; *b*: H_2O_2, $Na_2WO_4 \bullet 2H_2O$; *c*: *i*-PrONO; *d*: HCl/EtOAc; *e*: MeOH, $BF_3 \bullet Et_2O$; *f*: NBS, *hv*; *g*: potassium salt of Aspirin; *h*: potassium salt of Diclofenac; *i*: $(Boc)_2O$; *j*: *t*-BuOH, DCC, CuCl; *k*: $Ti(OBu\text{-}n)_4$; *l*: $2\text{-}AcOC_6H_4COCl$; *m*: CF_3COOH; *n*: MeOH, $Rh_2(OAc)_4$; *o*: HI; *p*: Zn, THF; *r*: PPh_3.

nitrite, displacement of diazogroup for chlorine or methoxy group, allyl bromination of the methyl group and the treatment of intermediate 3-bromomethyl cephalosporanates **3**, **4** with Aspirin or Diclofenac potassium salts.

Conversion of 7-ACA into **5**, **6** via intermediates **9** and **10** included Boc protection and esterification accompanied by the isomerization of the double bond into Δ2 position, hydrolysis of the 3-acetoxymethyl group with titanium *n*-butoxide, acylation of the hydroxyl group with 2-(acetoxy)benzoyl chloride, sulfur oxidation into sulfone and the double bond restoration into Δ3 position, deprotection of the amino group and its substitution for the chlorine or methoxy group via diazotation. This methodology was not so convenient as the first one: the acylation of **9** with acid chloride in the case of Diclofenac failed due to the intramolecular transformation of the latter into 1-(2,6-dichlorophenyl)-2-indolinone [10].

Transformation of 7-diazo intermediate **12** into 7α-iodo and 7,7-dihydrocephalosporanates **13**, **14** (sequence c) and insertion of triphenylphosphine into 3-bromomethyl cephalosporanate **3** (reaction) heading to **15**, were also performed.

3. Results and discussion

3.1. Basic hydrolysis and elastase inhibition

The hydrolytic splitting of the β-lactam ring of **5–8** at pH 7.3 and 37 °C monitored by HPLC demonstrated the liberation of Aspirin or Diclofenac from their 3-acyloxymethyl moiety according to the well known-mechanism [11], suggested the possibility of the parallel participation of these antiinflammatory drugs in the appropriate pathological process. The comparison of chemical half-lives for these compounds demonstrated the higher hydrolytic resistance of Diclofenac containing cephalosporanate 1,1-dioxides **7**, **8** than that of Aspirin containing **5**, **6** (see *table I*).

It has been shown that the mechanism of Elastase inhibition with cephalosporanate sulfones involves acylation of Ser-195 residue in enzyme active center [3]. In the case of deacetoxycephalosporins it leads only to the splitting of the β-lactam ring. 3-Acyloxymethyl derivatives of cephalosporins under these conditions are liberating carboxylic acid and transforming it into 3-exo methylene intermediate which could react with His-57 [3]. We have evaluated the difference in inhibiting intensity for these two structural types by measuring the amidolytic activity of Porcine Pancreas Elastase (Type III) in the presence of the synthesized compounds using a standard tetrapeptide *para*-nitroanilide as substrate.

The dependence of a steady state velocity on the inhibitor concentration was determined graphically with Hanes' and Dixon's plots [12]. Examples of noncompetitive inhibition mechanism patterns were obtained for deacetoxycephalosporins **1**, **2** and for DAC **6**, **8**. A mixed noncompetitive mechanism was observed for **7**. In the case of compounds **13**, **15**, **5** and **11** inhibition occurs according to competitive mechanism. The data in *table I* show that the values of the inhibitory effect of new DAC **5–8**, containing a leaving group, are characterized by a specificity greater than for deacetoxy derivatives **1**, **2**, **13** by two orders of magnitude; in this respect DAC **5–8** are comparable to *tert*-butyl 3-acetoxymethyl-7α-chlorocephalosporanate 1,1-dioxide (**11**) [3].

3.2. Antiinflammatory properties

Liberation of Aspirin and Diclofenac from the 3-acyloxymethyl moiety of DAC **5–8** leads predominantly to the involvement of these two inhibitors of prostaglandin biosynthesis in pathological process.

It is also known that a significant role in chronic inflammation of a mononuclear type belongs to macrophages which in the activated form regulate this process secreting different classes of compounds including neu-

Table I. Hydrolytic stability and elastase inhibitory properties of *tert*-butyl cephalosporanate sulfones.

Compound	Hydrolysis at pH 7.3 and 37 °C $t_{1/2}$ (h)	IC_{50} (mM) [a]	
		Dixon plot	Hanes plot
1		11.0 ± 0.9	10.4 ± 0.5
2		11.0 ± 0.2	10.2 ± 0.3
13		7.00 ± 0.03	13.5 ± 0.7
15		17.0 ± 0.1	15.2 ± 0.4
5	2.3	0.35 ± 0.03	0.20 ± 0.03
6	19		0.10 ± 0.02
7	73	0.68 ± 0.05	0.21 ± 0.02
8	60		0.10 ± 0.04
11 [3]		0.16 ± 0.02	0.18 ± 0.03

[a] $IC_{50} = K_i$ for **1**, **2**, **6**, **8**.

Table II. NO generation by RAW 264.7 macrophage cells in the presence of DAC. [a]

Concentration of tested compound (μg/mL)	NO concentration (mM/200 μL)							
	Nonactivated cells				Activated cells			
	5	**7**	Aspirin	Diclofenac	**5**	**7**	Aspirin	Diclofenac
0 (control)	4.0 ± 1.4	4.0 ± 1.1	5.0 ± 2.1	3.0 ± 1.6	14 ± 2.5	19 ± 1.7	17 ± 2.2	16 ± 3.6
1	8.0 ± 1.0	6.0 ± 1.3	n.t.	n.t.	39 ± 3.6	49 ± 6.2	n.t.	n.t.
10	10 ± 2.0	8.0 ± 1.1	3.0 ± 1.0	8.0 ± 2.0	47 ± 4.7	49 ± 6.2	20 ± 1.0	35 ± 2.0

[a] n.t.: not tested.

tral proteases and highly reactive nitric oxide molecules [13]. This means that expected antiinflammatary effect of synthesized DAC could be provided in three ways: (i) by the liberation of Aspirin or Diclofenac, (ii) by the inhibition of proteases and (iii) by the intracellular NO generation.

Table II demonstrates the effect of the addition of two DAC, Aspirin and Diclofenac to nonactivated and activated RAW 264.7 macrophage cells. It has been found that *tert*-butyl cephalosporanate 1,1-dioxides **5** and **7** intensifies NO generation in both types of macrophage cells by 2–3 times at 10 μg/mL concentration without any evidence of cell damage. In contrast to Aspirin which practically did not induce NO generation, Diclofenac was characterized by the same effect as cephalosporins.

3.3. Cytotoxic activity and nitric oxide radical generation in tumor and normal cells

According to recent investigations there is a strong assumption that the excessive induction of NO biosynthesis in cells could lead to their irreversible damage [13]. Potential cytotoxic activity of synthesized *tert*-butyl cephalosporanate sulfones based on such biological effect was tested in vitro on four 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 (TD_{50}) effect were determined according to the known procedure [14] 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.

The results of these experiments are summarized in *table III*. It can be seen that the majority of tested *tert*-butyl cephalosporanate 1,1-dioxides exhibited a cytotoxic potency in vitro both against tumor and normal cells. Substances **1**, **8** and **15** inhibited the tumor cells more effectively than the nomal ones but **3** and **7** exhibited the opposite properties. Such a differentiation could offer a way to initiate a search for cephalosporin analogs with more selective antitumor action. In total contrast to Diclofenac free Aspirin did not inhibit but only stimulated the proliferation of tumor cells.

In addition to the assays for evaluation of cytotoxic properties there was also measured the ability of the tested compounds to influence the NO biosynthesis in plate wells for each cell line. Total Generation ability parameter TG_{100} (see *table III*), representing this property extrapolated for 100% live cells in the presence of added substance was calculated according to the equation:

$$TG_{100} = G_{EX} \times 100/C \text{ (nM } 10^{-1}/200 \text{ μL)}$$

where: G_{EX} = concentration of NO (nM) in supernatant generated by surviving cells in 200 μL volume of plate cell after incubation with 50 μg/mL of a compound; C = percentage of the surviving cells after incubation with 50 μg/mL of a tested compound (CV: coloration).

The comparison of TG_{100} and TD_{50} for compounds with pronounced cytotoxic activity indicates a good correlation between these two parameters.

Preliminary analysis of structure–activity relationships for the cytotoxic action indicates the preference of the α configuration for the substituent at position C-7 but allowed broad variations at position C-3. Cephalosporonate sulfone derivatives with a free amino and carboxyl groups as well as unoxidized ones were characterized by the lack of cytotoxic and NO generating properties [15].

4. Conclusions

Synthesis and biological investigation of new *tert*-butyl cephalosporanate 1,1-dioxides containing in their acyloxymethyl moiety Aspirin and Diclofenac gave support to the assumption that the latter drugs are liberated

Table III. In vitro cell cytotoxicity and the ability of intracellular NO generation caused by cephalosporanate 1,1-dioxides.

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} (CV)	TD_{50} (MTT)	TG_{100}
1	6	6	450	6	2	200	3	2	633	2	2	500	46	29	1100
2	> 50	> 50	25	45	35	47	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
3	6	1	460	12	5	300	5	20	500	20	15	625	6	5	340
5	> 50	> 50	5	46	42	24	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
6	20	10	450	50	48	40	27	30	103	> 50	> 50	35	42	46	950
7	10	5	470	48	44	200	5	21	400	24	23	400	5	2	360
8	12	5	450	5	1	750	6	12	950	26	22	570	42	48	850
10	3	2	750	2	3	800	17	20	900	18	20	800	12	27	360
11	33	40	110	32	25	215	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
13	> 50	> 50	18	> 50	42	22	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
14	> 50	> 50	7	> 50	42	22	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
15	19	46	380	15	10	420	8	10	380	10	12	450	47	> 50	600
Aspirin	> 50	> 50	7	> 50	> 50	10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Diclofenac	38	22	30	25	22	35	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

[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 generation ability for 100% of surviving cells.

after splitting of the β-lactam ring by Elastase. It has been found that a few representatives of these compounds in low concentrations significantly increased the biosynthesis of nitric oxide in cells. In the case of macrophage the presence of DAC in the medium did not affect the survival of cells, but in tumor and normal cells lines the opposite effect was observed. These properties outline a new promising research area for *tert*-butyl cephalosporanate sulfones as a new type of dual antiinflammatory and antitumor agents.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Boetius PHMK 05 instrument and were 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 (λ = 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.

5.1.1. tert-Butyl 7α-chloro-3-methylceph-3-em-4-carboxylate 1,1-dioxide ***1***

25% Hydrogen peroxide (6.0 mL, 44 mmol) and $Na_2WO_4 \bullet 2H_2O$ (100 mg) were added slowly under stirring to a solution of *tert*-butyl 7β-amino-3-methylceph-3-em-4-carboxylate (2.35 g, 8.5 mmol) (prepared from 7-ADCA according to [9]) in acetonitrile (60 mL). The resulting mixture was stirred for 24 h at 4–10 °C, diluted with ethylacetate (200 mL), washed with water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The residue was dissolved in minimal volume of chloroform and after dilution with ether the white precipitate of *tert*-butyl 7β-amino-3-methylceph-3-em-4-carboxylate 1,1-dioxide (1.91 g, 74% yield) was collected by filtration and dried in vacuum desiccator over P_2O_5.

iso-Propylnitrite (0.8 mL, 8.0 mmol) was added under stirring to a solution of *tert*-butyl 7β-amino-3-methylceph-3-em-4-carboxylate 1,1-dioxide (1.50 g, 4.9 mmol) in dry methylene chloride (40 mL). The mixture was stirred for 1.5 h at room temperature and concentrated under reduced pressure. Purification by column chromatography with ethylacetate–hexane mixture (2:3) gave *tert*-butyl 7-diazo-3-methylceph-3-em-4-carboxylate 1,1-dioxide; m.p. = 124–126 °C. ^1H NMR ($CDCl_3$), δ: 1.55 (9H, s, C_4H_9); 2.09 (3H, s, CH_3); 3.64, 3.93 (2H, AB-q, J = 17, SO_2CH_2); 5.49 (1H, s, C_6-H). IR (nujol): 2100, 1760, 1720 cm^{-1}.

A solution of crude *tert*-butyl 7-diazo-3-methylceph-3-em-4-carboxylate 1,1-dioxide (1.77 g, 5.66 mmol) in ethylacetate

(20 mL) was treated with ethylacetate saturated with HCl (5 mL). The mixture was allowed to stand 30 min and concentrated under reduced pressure. The residue was crystallized from mixture of chloroform–ether giving **1** (1.11 g, 61% yield) (spectroscopic data identical with published ones [6]).

5.1.2. tert-Butyl 7α-methoxy-3-methylceph-3-em-4-carboxylate 1,1-dioxide **2**

Boron trifluoride etherate (0.62 mL, 4.9 mmol) was added under stirring to solution of *tert*-butyl 7-diazo-3-methylceph-3-em-4-carboxylate 1,1-dioxide (1.77 g, 5.66 mmol) (prepared as described above) in methanol (80 mL). The mixture was stirred for 24 h at room temperature and concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture 3:2 giving **2** (0.45 g, 25% yield) (spectroscopic data identical with published ones [6]).

5.1.3. tert-Butyl 3-bromomethyl-7α-chloroceph-3-em-4-carboxylate 1,1-dioxide **3** [6]

A solution of *tert*-butyl 7α-chloro-3-methylceph-3-em-4-carboxylate 1,1-dioxyde (**1**) (1.3 g, 4.04 mmol) and N-bromosuccinimide (NBS, 784 mg, 4.04 mmol) in benzene (200 mL) was irradiated and heated at reflux temperature with two 400 W incandescent lamps for 30 min, cooled, the volume was diminished 10 times by concentration. After standing for 4 h precipitated succinimide was filtered off and the filtrate was concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture 1:2 giving **3** (0.64 g, 40% yield); m.p. = 139–141 °C (from mixture: ether–petroleum ether). ^{1}H NMR ($CDCl_3$), δ: 1.58 (9H, s, C_4H_9); 3.82, 4.22 (2H, AB-q, J = 18, C_4-H_2); 4.17, 4.51 (2H, AB-q, J = 10, CH_2Br); 4.82 (1H, brs, C_6-H); 5.24 (1H, brs, C_7-H). Anal. $C_{12}H_{15}BrClNO_5S$. Found: C 35.57; H 3.75; N 3.29%. Calc.: C 35.97; H 3.77; N 3.50%.

5.1.4. tert-Butyl 3-bromomethyl-7α-methoxyceph-3-em-4-carboxylate 1,1-dioxide **4** [6]

This substance was prepared from **2** with 85% yield in the manner described above for **3**. ^{1}H NMR (DMSO-d_6), δ: 1.55 (9H, s, C_4H_9); 3.57 (3H, s, CH_3O); 3.73, 4.17 (2H, AB-q, J = 18, SO_2CH_2); 4.15, 4.44 (2H, s, AB-q, J = 10, CH_2Br); 4.73 (1H, brs, C_6-H); 5.15 (1H, d, J = 1, C_7-H).

5.1.5. tert-Butyl 3-(2-acetoxybenzoyloxy)methyl-7α-chloroceph-3-em-4-carboxylate 1,1-dioxide **5**

Method A: A solution of *tert*-butyl 3-bromomethyl-7α-chloroceph-3-em-4-carboxylate 1,1-dioxide (**3**) (4.0 g, 0.01 mol) and potassium 2-acetoxybenzoate (2.18 g, 0.01 mol) in acetonitrile (50 mL) was stirred and heated at 50 °C temperature for 20 h, cooled, diluted with ethylacetate (300 mL), washed with water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture (1:3) giving **5** (1.30 g, 26%). m.p. = 168–169 °C. Anal. $C_{21}H_{22}ClNO_9S$. Found: C 50.31; H 4.76; N 2.79%. Calc.: C 50.45; H 4.43; N 2.80%. ^{1}H NMR ($CDCl_3$), δ: 1.60 (9H, s, C_4H_9); 2.33 (3H, s, CH_3); 3.73, 4.11 (2H, AB-q, J = 19, SO_2CH_2); 4.75, 5.48 (2H, s, AB-q, J = 13, CH_2OCO); 4.91 (1H, s, C_6-H); 5.28 (1H, s, C_7-H); 7.06–8.11 (4H, m, C_6H_4). IR (nujol) 1800, 1770, 1760, 1730, 1720 cm^{-1}.

Method B: 2-Acetoxybenzoyl chloride (0.35 g, 1.75 mmol) in methylene chloride (3 mL) was added slowly under stirring to a solution of *tert*-butyl 3-hydroxymetyl-7β-*tert*-butoxycarbonylaminoceph-2-em-4-carboxylate (**9**) (0.58 g, 1.5 mmol; prepared from 7-ACA according to [8]) and triethylamine (0.24 mL, 1.75 mmol) in methylene chloride (20 mL). The resulting mixture was stirred for 24 h at room temperature, diluted with ethylacetate (200 mL), washed with 5% HCl, 5% $NaHCO_3$, water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The obtained residue (0.5 g) was analyzed comparing integration of ^{1}H NMR signals at 6.47 ppm (1H, s, SCH=) and at 3.55, 3.64 ppm (2H, AB-q, J = 18, SCH_2), and proved to be a mixture of *tert*-butyl 7β-*tert*-butoxycarbonylamino-3-(2-acetoxybenzoyloxy) methylceph-2-em-4-carboxylate and *tert*-butyl 7β-*tert*-butoxycarbonylamino-3-(2-acetoxybenzoyloxy)methylceph-3-em-4-carboxylate in a 2:1 ratio. The residue was then dissolved in acetonitrile (15 mL) and treated with 25% hydrogen peroxide (1.00 mL, 11 mmol) and Na_2WO_4•$2H_2O$ (40 mg). The mixture was stirred for 24 h at room temperature, diluted with ethylacetate (200 mL), washed with water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture (1:2) giving *tert*-butyl 7β-*tert*-butoxycarbonylamino-3-(2-acetoxybenzoyloxy)methylcephalosporanate 1,1-dioxide (**10**) (0.58 g, 67%); m.p. = 184–185 °C. Anal. $C_{26}H_{32}N_2O_{11}S$•$0.5H_2O$. Found: C 55.05; H 5.58; N 4.84%. Calc.: C 55.31; H 5.71; N 4.96%. ^{1}H NMR ($CDCl_3$), δ: 1.44 (9H, s, C_4H_9OCONH); 1.55 (9H, s, $COOC_4H_9$); 2.31 (3H, s, CH_3); 3.66, 4.04 (2H, AB-q, J = 19, SO_2CH_2); 4.89, 5.53 (2H, AB-q, J = 15, CH_2O); 4.89 (1H, d, J = 4, C_6-H); 5.71–5.74 (2H, m, C_7-H, NH); 7.02–8.00 (4H, m, C_6H_4).

The solution of **10** (580 mg, 1.0 mmol) in trifluoroacetic acid (6 mL) was allowed to stand 7 min at room temperature. The mixture was diluted with water, the precipitate was filtered off, washed with water and dried in vacuum desiccator over P_2O_5 giving *tert*-butyl 7β-amino-3-(2-acetoxybenzoyloxy)methylcephalosporanate 1,1-dioxide trifluoroacetate (232 mg, 38%). ^{1}H NMR (DMSO-d_6), δ: 1.49 (9H, s, $COOC_4H_9$); 2.29 (3H, s, CH_3); 4.20, 4.44 (2H, AB-q, J = 18, SO_2CH_2); 4.77, 5.22 (2H, AB-q, J = 13, CH_2O); 5.13 (1H, d, J = 4, C_6-H); 5.31 (1H, d, J = 4, C_7-H); 7.13–8.06 (4H, C_6H_4).

iso-Propylnitrite (0.05 mL, 0.5 mmol) was added under stirring to solution of *tert*-butyl 7β-amino-3-(2-acetoxybenzoyloxy) methylcephalosporanate 1,1-dioxide trifluoroacetate (140 mg, 0.23 mmol) in methylene chloride (10 mL). The mixture was stirred for 1.5 h at room temperature and concentrated under reduced pressure. Crude *tert*-butyl 7-diazo-3-(2-acetoxybenzoyloxy)methylceph-3-em-4-carboxylate 1,1-dioxide without purification was dissolved in ethylacetate (20 mL) and treated with ethylacetate saturated with HCl (0.5 mL). The mixture was allowed to stand 30 min and concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture (1:3) giving **5** (60 mg, 52%).

5.1.6. tert-Butyl 3-(2-acetoxybenzoyloxy)methyl-7α-methoxyceph-3-em-4-carboxylate 1,1-dioxide **6**

Method A: Cephalosporanate **6** was prepared from **4** in the manner described above for **5**. Ethylacetate–hexane mixture (1:4) was used for the fractionation of the reaction mixture by column chromatography giving the target substance with 36% yield, 93.9% purity according to HPLC (Lichrosorb RP8 and 0.2 M pH 5.0 am-

monium acetate buffer and acetonitrile 45:55 mixture as mobile phase). m.p. = 68–70 °C. ^{1}H NMR ($CDCl_3$), δ: 1.55 (9H, s, C_4H_9); 2.33 (3H, s, CH_3); 3.57 (3H, s, CH_3O); 3.68, 4.11 (2H, AB-q, J = 17, SO_2CH_2); 4.80 (1H, s, C_6-H); 4.80, 5.38 (2H, AB-q, J = 18, CH_2OCO); 5.17 (1H, s, C_7-H); 7.06–8.11 (4H, m, C_6H_4). IR (nujol) 1790, 1770, 1720 cm^{-1}.

Method B: *tert*-Butyl 7-diazo-3-(2-acetoxybenzoyloxy) methylceph-3-em-4-carboxylate 1,1-dioxide was converted into **6** with 27% yield in methanol in the presence of rhodium(II) acetate in the manner described in [3].

5.1.7. tert-Butyl 3-[2-(2,6-dichlorophenylamino)phenylacetoxymethyl]-7α-chloro-ceph-3-em-4-carboxylate 1,1-dioxide 7

A solution of *tert*-butyl 3-bromomethyl-7α-methoxyceph-3-em-4-carboxylate 1,1-dioxide (**4**) (2.00 g, 50 mmol) and potassium 2-(2,6-dichlorophenylamino)phenyl acetate (1.67 g, 50 mmol) in acetonitrile (50 mL) was stirred and heated at 50 °C temperature for 20 h, cooled, diluted with ethylacetate (300 mL), washed with water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture (1:3) giving **7** (0.62 g, 20%). m.p. = 65–67 °C. Anal. $C_{26}H_{25}Cl_3N_2O_7S$. Found: C 51.05; H 4.18; N 4.28%. Calc.: C 50.71; H 4.09; N 4.55%. ^{1}H NMR ($CDCl_3$), δ: 1.55 (9H, s, C_4H_9); 3.51, 3.78 (2H, AB-q, J = 18, SO_2CH_2); 3.82 (2H, s, $CH_2C_6H_4$); 4.73, 5.29 (2H, s, AB-q, J = 14, CH_2OCO); 4.71 (1H, s, C_6-H); 5.29 (1H, s, C_7-H); 6.33–7.66 (8H, m, C_6H_4, C_6H_3, NH). IR (nujol) 3330, 1810, 1725 cm^{-1}.

5.1.8. tert-Butyl 3-[2-(2,6-dichlorophenylamino)phenylacetoxymethyl]-7α-methoxyceph-3-em-4-carboxylate 1,1-dioxide 8

This compound was prepared from **4** in the manner described above for **7**. Ethylacetate–hexane mixture (1:4) was used for fractionation of the reaction mixture using column chromatography giving the target substance with 16% yield, 95.2% purity according to HPLC (Zorbax Pro-10 C_8 and 0.2 M pH 5.0 ammonium acetate buffer and acetonitrile 40:60 mixture as mobile phase). m.p. = 48–50 °C. ^{1}H NMR ($CDCl_3$), δ: 1.55 (9H, s, C_4H_9); 3.55 (3H, s, CH_3O); 3.66, 3.88 (2H, AB-q, J = 13, SO_2CH_2); 3.77 (2H, s, $OCOCH_2Ph$); 4.82 (1H, s, C_6-H); 4.82, 5.17 (2H, s, AB-q, J = 18, CH_2OCO); 5.15 (1H, s, C_7-H); 6.33–6.66 (2H, m, NH, C_6H); 6.93–7.62 (6H, m, C_6H_4, C_6H_3). IR (nujol) 3330, 1800, 1720 cm^{-1}.

5.1.9. tert-Butyl 7α-iodo-3-methylceph-3-em-4-carboxylate 1,1-dioxide 13

The crude *tert*-butyl 7α-diazo-3-methylceph-3-em-4-carboxylate 1,1-dioxide (310 mg, 0.01 mol) was dissolved in methylene chloride (50 mL) and gaseous HI (0.03 mol) was bubbled through solution at –20 °C during 30 min. The mixture was heated till room temperature, washed with 1 N $Na_2S_2O_3$, water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The residue was fractionated by column chromatography with ethylacetate–hexane mixture (1:1) giving **13** (2.31 g, 56%). m.p. = 115–117 °C. Anal. $C_{12}H_{16}INO_5S•0.5EtOAc$. Found: C 36.86; H 4.08; N 3.34%. Calc.: C 36.77; H 4.40; N 3.06%. ^{1}H NMR ($CDCl_3$), δ: 1.54 (9H, s, C_4H_9); 2.06 (3H, s, CH_3); 3.62, 3.89 (2H, AB-q, J = 19, SO_2CH_2); 4.80 (1H, d, J = 2, C_6-H); 5.38 (1H, s, C_7-H). IR (nujol) 1775, 1710 cm^{-1}.

5.1.10. tert-Butyl 7,7-dihydro-3-methylceph-3-em-4-carboxylate 1,1-dioxide 14

Zinc powder (220 mg) was added to solution of **13** (450 mg, 1.1 mmol) in a mixture of tetrahydrofuran (4 mL) and 1 M aqueous ammonium acetate (2 mL). Suspension was stirred 2 h at room temperature, diluted with diethyl ether (50 mL), filtered and the filtrate was washed with water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The residue was crystallized from hexane giving **14** (170 mg, 51%). m.p. = 183–185 °C. Anal. $C_{12}H_{17}NO_5S•0.5H_2O$. Found: C 48.91; H 5.85; N 4.85%. Calc.: C 48.63; H 6.12; N 4.72%. ^{1}H NMR ($CDCl_3$), δ: 1.51 (9H, s, C_4H_9); 2.02 (3H, s, CH_3); 3.48 (2H, m, C_7-H); 3.55, 3.89 (2H, AB-q, J = 19, SO_2CH_2); 4.68 (1H, m, C_6-H). IR (nujol) 1720, 1770 cm^{-1}.

5.1.11. tert-Butyl 3-(triphenylphosphonium)methyl-7α-chloroceph-3-em-4-carboxylate 1,1-dioxide bromide 15

Triphenylphosphine (654 mg, 25 mmol) was added under stirring to solution of *tert*-butyl 3-bromomethyl-7α-chloroceph-3-em-4-carboxylate 1,1-dioxide (**3**) (1. 0 g, 25 mmol) in acetonitrile (30 mL). The mixture was allowed to stand for 24 h and was filtrated giving **15** (1.01 g, 51%). m.p. = 185–186 °C. Anal. $C_{30}H_{30}BrClNO_5PS•H_2O$. Found: C 52.87; H 4.63; N 2.10%. Calc.: C 52.90; H 4.70; N 2.00%. ^{1}H NMR (DMSO-d_6), δ: 1.11 (9H, s, C_4H_9); 4.22 (2H, brs, SO_2CH_2); 4.89, 5.07 (2H, brs, brs, CH_2P); 5.75 (1H, brs, C_6-H); 5.86 (1H, d, J = 1, C_7-H); 7.55–8.11 (15H, m, $3C_6H_5$). IR (nujol) 1810, 1740, 1715 cm^{-1}.

5.2. Hydrolytic stability of cephalosporins

Tested compounds were dissolved in 0.05 M pH 7.4 phosphate buffer, containing dimethylsulfoxide 10–20% as solubilizing vehicle in the following concentrations: **5**: 0.20 mg/mL, **6**: 1.00 mg/mL, **7**: 0.11 mg/mL, **8**: 0.20 mg/mL, and thermostated at 37 °C. Hydrolysis of substances was monitored by HPLC using 5 μm Silasorb SPH C_{18} cartridge (150 × 4.7 mm) and 0.2 M pH 5.0 ammonium acetate buffer and acetonitrile 1:1 mixture as mobile phase. Chemical half-lives for compounds **5–8** (*table 1*) were determined from the first-order kinetic analysis of time-dependent decrease of their parent peak areas.

Aspirin and Diclofenac in the identical phosphate buffer–dimethylsufoxide mixtures were used as reference substances in the HPLC analysis of hydrolytic degradation cephalosporanate 1,1-dioxides containing these drugs in precursor form.

5.3. Inhibition of porcine pancreas elastase

The inhibiting effect was determined according to a standard method adapted on 96 wells plates measuring optical density. Total volume 180 mL in each well consisted of: (a) 150 mL N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide solution in concentrations of 2.0, 1.0, 0.5 and 0.25 mM dissolved in 0.1 mM Hepes (pH 7.5) and 10% DMSO; (b) 5 mg of the Porcine Pancreas Elastase (Type III) dissolved in 10 mL 0.1 M phosphate buffer pH 7.4; (c) 20 mL of the DMSO solution containing inhibitors **1**, **2**, **13** and **15** in 0.4, 1.2, 3.7 and 11 × 10^{-5} M concentrations, inhibitors **5–8**, **11** in 0.8, 2.5 and 7.4 × 10^{-7} M concentrations.

5.4. Nitric oxide generation in macrophage cells

The macrophage cell line RAW 264.7 (obtained from European Collection of Animal Cell Cultures) was cultivated in DMEM

standard medium without indicator and antibiotics. After the ampoule was defreezed the cells RAW 264.7 were cultivated for three days, removed from the carrier and then transferred in DMEM with the tested compounds. The cells (1×10^6 cells/mL) were then activated with 35 mg/mL LSP (lipopolysaccharide from *E. coli*) and 5 units/mL of recombinant murine INFg (interferon g). The activation was carried out in bottles. Activated cells were then placed into the plate hollows. Nonactivated macrophages with and without tested compounds served as control. Cell survival (more than 90%) in the process of the experiment was determined by the number of alive cells in colorimetric test with Neutral Red (NR) [14]. Registrations were made 18 h after activation was started. Concentration of NO was determined as NO_2 concentration in plate wells according to [16].

5.5. In vitro cytotoxicity assay

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 test compounds were diluted and added in wells to give the final concentrations of 50, 25, 12.5 and 6.25 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% [16, 17]. Concentration of NO was determined according to [16].

Acknowledgements

We are grateful to Taiho Pharmaceutical Co. and Gist-brocades for generous gifts of 7-ACA and 7-ADCA.

References

[1] Dax S.L., Antibacterial Chemotherapeutic Agents, Blackie Academic & Professional, London, 1997, pp. 74–157.

[2] Lamotte-Brasseur J., Knox J., Kelly J.A., Charlier P., Fonze E., Diderberg O., Frere J.-M., Biotechnol. Gen. Eng. Rev. 12 (1994) 189–230.

[3] Doherty J.B. et al., J. Med. Chem. 33 (1990) 2513–2521.

[4] Otani T., Oie S., Matsumoto H., Tempest M., Micetich R.G., Singh R., Yamashita T., PCT Int. Appl. WO 94 01109 (1974); Chem. Abstr. 121 (1994) 57497p.

[5] Arrigoni-Martelli E., in: Glynn L.E., Houck J.C., Weissmann J.C. (Eds.), Handbook of Inflammation, Vol. 5, Elsevier, Amsterdam, 1985, pp. 1–25.

[6] Alpegiani M., Baici A., Bissolino P., Carminati P., Cassinelli G., Nero S.D., Franceschi G., Orezzi P., Perrone E., Rizzo V., Sacchi V., Eur. J. Med. Chem. 27 (1992) 875–890.

[7] Blacklock T.J., Butcher J.W., Sohar P., Lamanec T.R., Grabowski E.J.J., J. Org. Chem. 54 (1989) 3907–3913.

[8] Veinberg G.A., Petrulanis L., Grigan N., Turovsky I., Musel D., Lukevics E., Khim. Heterotsikl. Soedin. (1996) 110–114.

[9] Grigan N., Musel D., Veinberg G.A., Lukevics E., Synth. Commun. 26 (1996) 1183–1185.

[10] Geigy J.R., A.-G. Brit. Pat. (1968) 1132318; Chem. Abstr. 70 (1969) 57455b.

[11] Hamilton-Miller J.M.T., Newton G.G.F., Abraham E.P., Biochem. J. 116 (1970) 371–395.

[12] Wong J.T.-F., Kinetics of Enzyme Mechanisms, Academic Press, London, 1975.

[13] Kerwin Jr. J.F., Lancaster Jr. F.R., Feldman P.L., J. Med. Chem. 38 (1995) 4343–4362.

[14] Riddell R.J., Clothier R.H., Balls M.Fd., Chem. Toxicol. 24 (1986) 469–471.

[15] Unpublished results.

[16] Fast D.J., Lynch R.C., Leu R.W., J. Leuckocyt. Biol. 52 (1992) 255–261.

[17] Freshney P.J., Culture of Animal Cells (A Manual of Basic Technique), Wiley-Liss, New York, 1994, pp. 296–297.