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SYNTHESIS AND BIOLOGICAL ACTIVITY OF ALKYLIDENE-SUBSTITUTED CEPHEMS AND PENAMS

I. Potorocina[1], M. Vorona[1], I. Shestakova[1], I. Domracheva[1], E. Liepinsh[1], and G. Veinberg[1]*

The condensation of tert-butyl esters of 3-methyl-7-oxoceph-3-em-4-carboxylic and 6-oxopenicillanic acids with a series of 2-oxoalkylidene(triphenyl)phosphoranes gave tert-butyl esters of new cephalosporin and penicillin analogs with an alkylidene substituent in the β-lactam ring. Most of these products were oxidized by meta-chloroperbenzoic acid to the corresponding sulfones. The cephemes and penams synthesized including the oxidized products displayed high cytotoxicity relative to cancer cells in vitro. Some of the alkylidene-substituted cephems as the free acids, similar to Tazobactam, inhibit the catalytic activity of Enterobacter cloacae penicillinase.

Keywords: *tert*-butyl esters of (6*Z*)-alkylidene-1,1-dioxopenicillanic acids, *tert*-butyl esters of (7*Z*)-alkylidene-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acids, (6*Z*)-(N-methyl-N-methoxycarbamoylmethylidene)-1,1-dioxopenicillanic acid, 3-methyl-(7*Z*)-(N-methyl-N-methoxycarbamoylmethylidene)-1,1-dioxoceph-3-em-4-carboxylic acid, cyclic sulfones, condensation, oxidation by *m*-chloroperbenzoic acid.

Continuing a study of the relations between structure and biological activity of cephalosporin and penicillin derivatives [1], we report here the synthesis of new analogs of these antibiotics with an alkylidene substituent in the β-lactam ring as well as their 1,1-dioxo derivatives and the anticancer activity of these compounds *in vitro*. The effect of the *tert*-butyl carboxyl protective group on the capacity of some of these cephalosporin and penicillin analogs to inhibit β-lactamase was clarified.

The desired *tert*-butyl esters of 7-alkylidene-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acids **3a–c** were synthesized by condensation of the *tert*-butyl ester of 7-oxo-3-methylceph-3-em-4-carboxylic acid (**1**) with N-methoxycarbamoyl-N-methylmethylidene- (**2a**), 3-ethoxycarbonyl-2-oxopropylidene- (**2b**), and 2-(2-furyl)-2-oxoethylidene(triphenyl)phosphorane (**2c**), respectively. Products **3a–c** were oxidized using *meta*-chloroperbenzoic acid (MCPBA) to give the corresponding sulfones **4a–c.**

*To whom correspondence should be addressed, e-mail: veinberg@osi.lv.

Latvian Institute of Organic Synthesis, 21 Aizkraukles St., Riga LV-1006 , Latvia.

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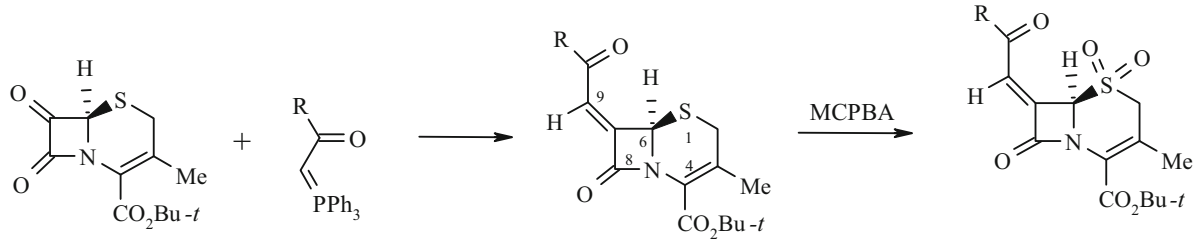
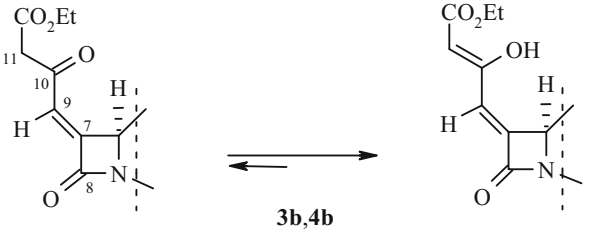


1 **2a–c** **3a–c** **4a–c**

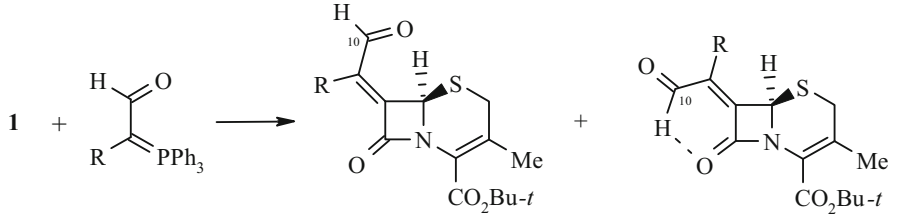
2–4 a R = N(OMe)Me, **b** R = CH_2COOEt, **c** R = 2-furyl

The ^{1}H NMR spectral data for **3a,c** and **4 a,c** indicate that their alkylidene substituent has (7*Z*)-configuration: the singlet for H-9 proton in these compounds is found at 6.6–7.2 ppm and is characteristic for (7*Z*)-methylidenecephalosporins [2, 3]. The signals of the alkylidene substituent protons in **3b** and **4b**, which can undergo keto-enol tautomerization, are seen as three one-proton singlets at 5.4–5.5, 6.4–6.6, and 11.7–11.8 ppm. The signal for the 11-CH_2 group protons is lacking, while the signal for H-9 proton is shifted upfield relative to the analogous signal in the spectra of **3a,c** and **4a,c.** These features suggest a predominantly enol form for this substituent in $CDCl_3$ solution.



3b,**4b**

The condensation of ester **1** with 1-formylmethylidene- (**5a**) and 1-formylethylidene(triphenyl)phosphorane (**5b**) did not proceed with such high stereospecificity. The reaction products, namely, the *tert*-butyl esters of 3-methyl-7-(1-formylmethylidene)- (**6a**) and 3-methyl-7-(1-formyl-1-ethylidene)ceph-3-em-4-carboxylic acids (**6b**), were obtained as 3:2 mixtures of the (7*Z*) and (7*E*) isomers as indicated by ^{1}H NMR spectroscopy. The mixtures were separated by preparative column chromatography.

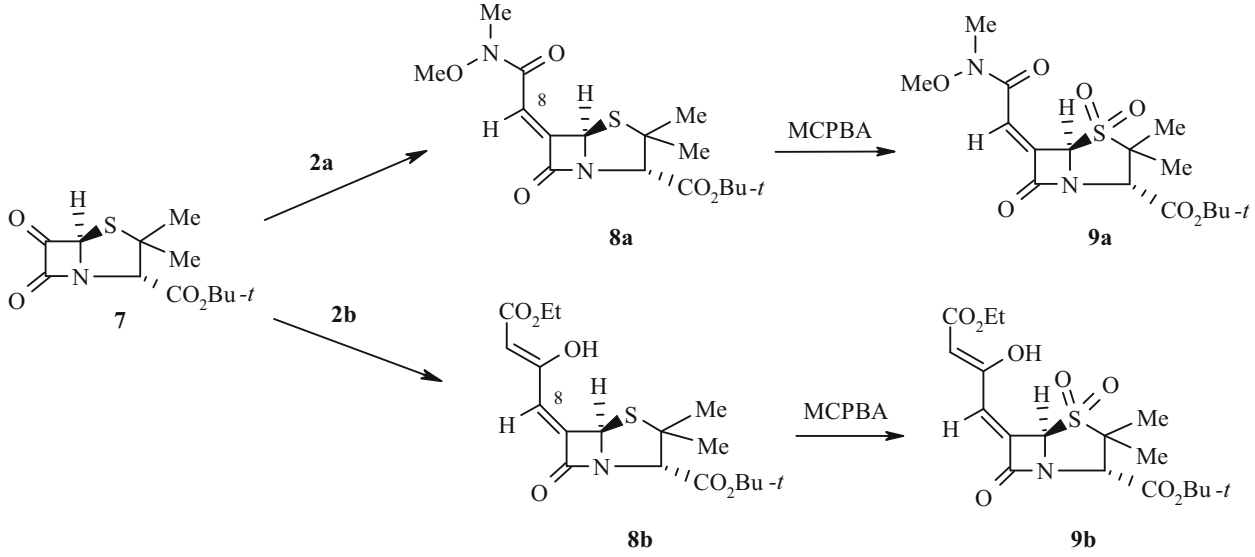


5a,b (7*Z*)-**6a,b** (7*E*)-**6a,b**

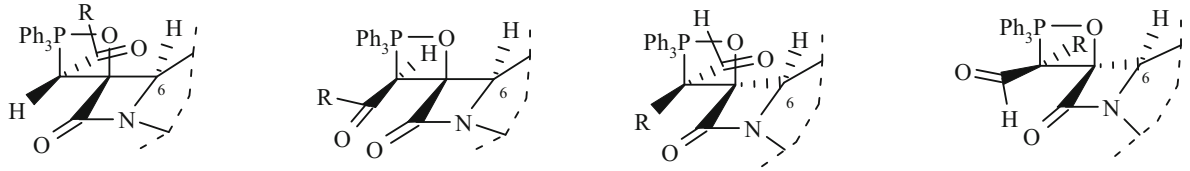
5, 6 a R = H, **b** R = Me

The position of aldehydic proton H-10 is a distinguishing feature in the ^{1}H NMR spectra of (7*E*)-isomers **6a** and **6b**. This signal is shifted downfield relative to the analogous signal of the (*Z*)-isomers (by 0.51 ppm for ester **6a** and 0.63 ppm for ester **6b**) due to hydrogen bonding between H-10 proton and the lactam ring C=O group.

Condensation of the *tert*-butyl ester of 6-oxopenicillanic acid (**7**) with phosphoranes **2a** and **2b** led to the formation of *tert*-butyl esters of 6-alkylidenepenicillanates **8a** and **8b** with predominantly (*Z*)-configuration of the alkylidene fragment and its enolic form in product **8b**. Oxidation of these products using MCPBA gave the corresponding sulfones **9a** and **9b**.



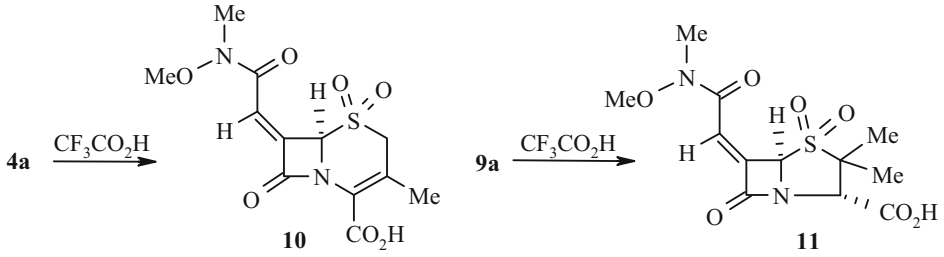
The steric specificity discovered for the condensation of cephem **1** and penam **7** with phosphoranes **2a–2c** and the lack of such specificity in the analogous reactions of cephem **1** with phosphoranes **5a** and **5b** probably should be attributed to differences in the structures responsible for formation of intermediate [2+2] cycloaddition products according to the mechanism of the Wittig reaction [4]. In the case of phosphoranes **2a–c** there is possible formation of forms **A** and **B** or in the case of phosphoranes **5a** and **5b** – forms **C** and **D**. These intermediate forms determine the configuration of the final products.



Thus, the virtually quantitative formation of cephems **3a–c** and penams **8a** and **8b** in the (7*Z*)-isomeric form may be attributed to the predominant formation of form **A** due to mutual repulsion of the C=O substituent in the oxaphosphoethane ring and C=O group of the lactam ring in form **B**.

The formation of 7-(1-formylmethylidene)cephems **6a** and **6b** as a 3:2 mixture of the (7*Z*)- and (7*E*)-isomers as indicated by ^{1}H NMR spectroscopy suggests a competing effect of hydrogen bonding between the formyl group carbonyl and H-6 proton of the azetidine ring in form **C** and also between the formyl group proton and β-lactam carbonyl in form **D**.

Cleavage of the ester bond using trifluoroacetic acid in cephem **4a** and penam **9a** gave acids **10** and **11**, respectively.



Attempts to use a solution of $ZnBr_2$ in dichloromethane [5] for the analogous cleavage of esters **4a** and **9a** led to the formation of unidentified products mixture.

Evaluation of the cytotoxic activity of the synthesized cephems *in vitro* entailed its determination relative to monolayer human fibrosarcoma cell lines HT-1080 and murine hepatoma cell lines MG22A in comparison with the analogous activity relative to normal embryonic murine fibroblast cells 3T3. Staining of the 3T3 fibroblasts with neutral red permitted calculation of the expected toxicity LD_{50} for the compounds tested using a correlation equation developed at the US National Institutes of Health without recourse to *in vitro* experiments in the initial state of biological testing [6].

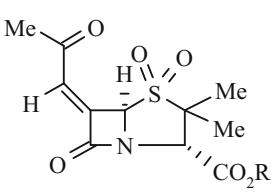
The screening data for cephems **3a,b, 4a–4c**, (7*Z*)-**6b**, and (7*E*)-**6b** (Table 1) showed that these compounds have high and virtually identical cytotoxicity relative to cancer cells (independently of the structure of the alkylidene fragment, its configuration, and extent of oxidation of the sulfur atom) and the capacity to generate NO radicals in the cell medium, which inhibit division of the cancer cells (as the result of a chemical reaction with their proteins and nucleic acids [7]). Moderate toxicity was found for cephem **3a**, its sulfone **4a**, and sulfone **4c**, while identically high toxicity was found for the (7*Z*)- and (7*E*)-isomers of cephem **6a**.

Bacterial β-lactamases from *Bacillus cereus* and *Enterobacter cloacae* were used to evaluate the inhibiting properties of the synthesized alkylidenecephems and alkylidenepenams with esterified and free carboxylic acid groups as well as of 2-oxopropylidenepenams **12a,2b** and 2-oxopropylidenecephems **13a,b** [1, 8, 9].

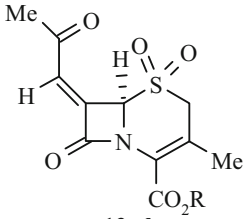
TABLE 1. Cytotoxic Properties of *tert*-Butyl Esters of Alkylidenecephems

Compound	IC_{50}, μg/ml*							LD_{50}, mg/kg
	HT-1080			MG-22A			3T3	
	CV	MTT	TG	CV	MTT	TG	NR	
3a	3	3	100	3	3	100	19	425
3b	2	3	67	3	3	50	15	343
4a	2	2	100	2	3	100	14	383
4b	4	4	250	3	3	250	10	331
4c	2	4	46	3	3	41	18	395
(7*Z*)-**6a**	1	9	83	3	3	43	4	177
(7*E*)-**6a**	2	2	100	1	1	60	4	177
(7*Z*)-**6b**	3	3	40	3	3	100	10	278
(7*E*)-**6b**	3	3	67	3	3	100	17	371

*IC_{50} is the concentration providing the annihilation of 50% of cells, CV is the staining with crystal violet, MTT is staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, TG is the specific NO generating activity of the compound, NR is staining with neutral red, and LD_{50} is the calculated expected toxicity.

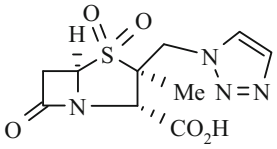


12a,b

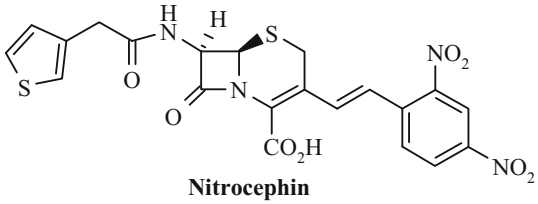


13a,b

a R = H, **b** R = *t*-Bu



Tazobactam



Nitrocephin

Prior to the addition of the standard Nitrocefin substrate, penicillinase was incubated with the tested compound for 10 min. The inhibiting action of the compounds and of the standard Tazobactam inhibitor was determined spectrophotometrically relative to the change in intensity of absorption at 486 nm over 5 min in comparison with the control consisting of nitrocefin and β-lactamase solution.

The testing of the synthesized compounds showed that they all lack inhibiting properties relative to *Bacillus cereus* penicillinase in contrast to tazobactam, which lowered the catalytic activity of this enzyme by 50% at a concentration of 0.36 μM.

The screening data relative to *Enterobacter cloacae* penicillinase given in Table 2 show that acids **10** and **13a**, in contrast to their *tert*-butyl esters **4a** and **13b**, reduce the catalytic activity of this enzyme by 50% in concentrations of 14.4 and 8.2 μg/ml comparable with Tazobactam at a concentration of 3.3 μg/ml.

Thus, (6*Z*)-(N-methoxycarbamoyl-N-methyl methylidene)-1,1-dioxopenicillanic acid **11** and its *tert*-butyl ester **9a** display a weak inhibiting effect only in concentrations of 200 μg/ml. The *tert*-butyl esters of alkylidenecephalosporanates **4b**, (7*Z*)-**6b**, (7*E*)-**6b**, and penicillanates **9a** and **12b** as well as penicillanic acid **12a** lack an inhibiting effect.

These results show that the *tert*-butyl esters of 7-alkylidene-3-methyl-1,1-dioxoceph-3-em-4-carboxylic acids, which possess anticancer activity, may be transformed into β-lactamase inhibitors upon their deesterification.

TABLE 2. Inhibiting Activity of Alkylidene-Substituted Cephems and Penams Relative to *Enterobacter cloacae* β-Lactamase

Conc., μg/ml*	Enzyme catalytic activity inhibition, %								
	4a	**10**	**13b**	**13a**	**9a**	**11**	**12b**	**12a**	Tazobactam
200	–15±0	82±4	–1±1	87±2	48±3	31±7	–9±5	13±3	100±3
50	–20±9	84±7	2±1	74±3	28±3	13±4	3±4	0±5	96±6
12.5	12±1	51±3	10±4	56±2	8±1	1±2	2±4	3±0	89±1
3.13	0±10	36±5	9±7	30±6	0±10	–9±10	1±3	3±3	44±3
0.78	4±3	23±3	4±7	18±3	–10±4	–8±5	2±3	7±9	12±1
0.2	7±1	29±11	9±1	6±2	–17±2	4±1	2±1	3±0	4±1
IC_{50}*	–	**14.4**	–	**8.2**	**200**	**>200**	–	–	**3.3**

*IC_{50} is the concentration providing for 50% inhibition of the catalytic activity of the enzyme.

EXPERIMENTAL

The ^{1}H NMR spectra were taken on a Varian Mercury-400 spectrometer at 400 MHz in $CDCl_3$ (for compounds **4a–c, 6a,b, 9a,b**) and DMSO-d_6 (for compounds **10** and **11**) with HMDS as internal standard. The ESI mass spectra were taken on a Micromass Quatro Micro™ API instrument in acetonitrile solution. The reaction course was monitored by thin-layer chromatography on Merck Kieselgel plates with development in UV light. Preparative column chromatography (CC) was carried out on Merck Kieselgel silica gel (0.060–0.200 mm) using a mixture of ethyl acetate (EA) and petroleum ether (PE) as the eluent. The reagents and materials were obtained from Acros and Aldrich.

The optical density in the biological tests in 96-well microtiter plates was determined using a Tetretek Multiscan MC C/340 horizontal spectrophotometer.

***tert*-Butyl Ester of (7*Z*)-(N-Methoxycarbamoyl-N-methylmethylidene)-3-methyl-1,1-dioxoceph-3-em-4-carboxylic Acid ((7*Z*)-4a).** Triphenylphosphorane **2a (**445 mg, 1.22 mmol) was added to a solution of ester **1** (330 mg, 1.22 mmol) in dichloromethane (20 ml) at 0°C. The mixture was stirred for 2 h at 0°C and then evaporated at reduced pressure. Column chromatography of the residue using 1:5 EA–PE as the eluent gave 198 mg (46%) of ***tert*-butyl ester of (7*Z*)-(N-methoxycarbamoyl-N-methylmethylidene)-3-methyl-ceph-3-em-4-carboxylic acid ((7*Z*)-3a)** as an oil; R_f 0.40 (1:1 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.55 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.09 (3H, s, CH_3); 3.17 and 3.56 (2H, two d, AB system, ^2J = 18.6, SCH_2); 3.28 (3H, s, NCH_3); 3.75 (3H, s, OCH_3); 5.51 (1H, s, H-6); 6.97 (1H, s, H-9). ESI-MS mass spectrum: Found: *m/z* 377. $C_{16}H_{22}N_2O_5S$. Calculated: M = 354. $[M+Na]^+$ = 377.

70% MCPBA (238 mg, 1.38 mmol) was added to a solution of ester (7*Z*)-**3a** (195 mg, 0.55 mmol) in dichloromethane (20 ml) at 0°C under stirring. The mixture was stirred at room temperature for 16 h, diluted by 20 ml dichloromethane adding, washed with 50 ml 5% aqueous sodium sulfite, two 50 ml portions of 5% aqueous sodium carbonate, and dried over anhydrous sodium sulfate. The solvent was evaporated at reduced pressure. Column chromatography of the residue using 1:5 EA–PE as the eluent gave 165 mg (78%) of desired ester (7*Z*)-**4a;** R_f 0.15 (1:1 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.53 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.11 (3H, s, CH_3); 3.28 (3H, s, NCH_3); 3.76 (3H, s, OCH_3); 3.69 and 3.99 (2H, two d, AB system, ^2J = 17.6, SCH_2); 5.70 (1H, s, H-6); 7.19 (1H, s, H-9). ESI-MS mass spectrum: Found: *m/z* 386. $C_{16}H_{22}N_2O_7S$. Calculated: M = 386. Found, %: C 49.92; H 5.49; N 6.87. $C_{16}H_{22}N_2O_7S$. Calculated, % : C 49.73; H 5.74; N 7.25.

***tert*-Butyl Ester of (7*Z*)-(3-Ethoxycarbonyl-2-hydroxyprop-2-enylidene)-3-methyl-1,1-dioxo-ceph-3-em-4-carboxylic acid ((7*Z*)-4b).** A solution of sodium hydroxide (30 mg, 0.75 mmol) in water (5 ml) was added with stirring to a solution of 3-ethoxycarbonyl-2-oxopropylidene(triphenyl)phosphonium chloride (317 mg, 0.743 mmol) in dichloromethane (10 ml) at 0°C. The solution was stirred for 30 min at 10°C. The organic phase containing phosphorane **2b** was separated, dried over sodium sulfate, and then added to a solution of 200 mg (0.743 mmol) ester **1** in 20 ml dichloromethane. The reaction mixture was stirred for 2 h at 0°C, then solvent was evaporated at reduced pressure. Column chromatography of the residue with 1:5 EA–PE as the eluent gave 225 mg (80%) ***tert*-butyl ester of (7*Z*)-(3-ethoxycarbonyl-2-hydroxyprop-2-enylidene)-3-methyl-ceph-3-em-4-carboxylic acid ((*Z*)-3b)** as an oil; R_f 0.65 (1:3 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.30 (3H, t, *J* = 7.1, $CH_2C\underline{H}_3$); 1.55 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.07 (3H, s, CH_3); 3.16 and 3.55 (2H, two d, AB system, ^2J = 19.0, SCH_2); 42.4 (2H, q, *J* = 7.1, $C\underline{H}_2CH_3$); 5.32 (1H, s, H-6); 5.44 (1H, s, H-9); 6.39 (1H, s, H-11); 11.76 (1H, s, OH). ESI-MS mass spectrum: Found: *m/z* 404. $C_{18}H_{23}NO_6S$. Calculated: M = 381. $[M+Na]^+$ = 404.

According to the procedure for the synthesis of ester (7*Z*)-**4a**, a solution of ester (7*Z*)-**3b** (225 mg, 0.59 mmol) in dichloromethane (20 ml) was oxidized using 70% MCPBA (255 mg, 1.48 mmol) for 4 h. After work-up of the reaction mixture, column chromatography of the residue with 1:4 EA–PE as the eluent gave 112 mg (46%) ester (7*Z*)-**4b**; R_f 0.36 (1:2 EA–PE); mp 136–140°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.30 (3H, t, *J* = 7.1, $C\underline{H}_3CH_2$); 1.53 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.10 (3H, s, CH_3); 3.68 and 3.90 (2H, two d, AB system, ^2J = 17.4, SCH_2); 4.24 (2H, q, *J* = 7.1, $C\underline{H}_2CH_3$); 5.37 (1H, s, H-6); 5.51 (1H, s, H-9); 6.62 (1H, s, H-11); 11.67 (1H, s, OH). Found, %: C 49.94; H 5.66; N 3.52. $C_{18}H_{23}NO_8S\cdot H_2O$. Calculated, %: C 50.10; H 5.84; N 3.25.

***tert*-Butyl Ester of (7*Z*)-[2-(2-Furyl)-2-oxoethylidene]-3-methyl-1,1-dioxoceph-3-em-4-carboxylic Acid ((7*Z*)-4c).** A solution of NaOH (0.3 g, 7.5 mmol) in water (20 ml) was added with stirring to a solution of 2-(2-furyl)-2-oxoethylidene(triphenyl)phosphonium bromide (3.25 g, 7.2 mmol) in dichloromethane (20 ml) at 0°C. The solution was stirred for 30 min at 10°C, then extracted with ethyl acetate, and dried over sodium sulfate. The extract containing phosphorane **2c** was evaporated and the residue was dissolved in dichloromethane (10 ml) and added to the solution of ester **1** (1.94 g, 7.2 mmol) in dichloromethane (20 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C and then evaporated. Column chromatography of the residue using 1:5 EA–PE as the eluent gave an oily product with R_f 0.31 (1:3 EA–PE), which was dissolved in dichloromethane (20 ml). Then, 70% MCPBA (4.4 g, 18 mmol) was added to the obtained solution under stirring. The reaction and work-up of the reaction mixture were carried out as described above for ester **4b**. After evaporation, column chromatography of the residue using 1:3 EA–PE as the eluent gave 1.24 g (44%) amorphous ester (7*Z*)-**4c**, R_f 0.30 (1:1 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.54 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.13 (3H, s, CH_3); 3.70 and 3.95 (2H, dd, AB system, ^2J = 17.6, SCH_2); 5.69 (1H, br. s, H-6); 6.61–6.67 (1H, m, H-9); 7.42 (1H, d, ^3J = 4.4, H furyl); 7.56 (1H, d, ^3J = 1.5, H furyl); 7.70 (1H, m, H furyl). ESI-MS mass spectrum: Found: *m/z* 394. Calculated: M = 393. $[M+H]^+$ = 394. Found, %: C 54.77; H 4.88; N 3.71. $C_{18}H_{19}NO_7S$. Calculated, %: C 54.95; H 4.87; N 3.56.

***tert*-Butyl Ester of (7*E*)-(1-Formylmethylidene)-3-methylceph-3-em-4-carboxylic acid ((7*E*)-6a)** and ***tert*-Butyl Ester of (7*Z*)-(1-Formylmethylidene)-3-methylceph-3-em-4-carboxylic acid ((7*Z*)-6a).** Employing the procedure for the preparation of ester (7*Z*)-**4b**, a solution of 1-formylmethylidene(triphenyl)phosphonium chloride (222 mg, 0.65 mmol) in dichloromethane (10 ml) was treated with a solution of sodium hydroxide (26 mg, 0.65 mmol) in water (5 ml). The resulting solution of phosphorane **5a** in dichloromethane (10 ml) was added to ester **1** (175 mg, 0.65 mmol) in dichloromethane (20 ml). After evaporation, column chromatography of the residue using 1:5 EA–PE as the eluent gave 142 mg (74%) of the (7*E*- and (7*Z*)-isomers of ester **6a** mixture as an amorphous substance; R_f 0.57 (1:2 EA–PE). Found, %: C 56.73; H 5.83; N 4.71. $C_{14}H_{17}NO_4S$. Calculated, %: C 56.93; H 5.80; N 4.74.

This mixture of isomers was separated by column chromatography using 1:20 EA–PE as the eluent to give 49 mg (25%) (*E*)-isomer (7*E*)-**6a** and 71 mg (37%) (*Z*)-isomer (7*Z*)-**6a**.

Ester (7*E*)-**6a** was obtained as a crystalline compound; R_f 0.54 (1:2 EA–PE); mp 102–122°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.56 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.11 (3H, s, CH_3); 3.22 and 3.55 (2H, two d, AB system, ^2J = 18.2, SCH_2); 5.27 (1H, s, H-6); 6.22 (1H, d, ^3J = 7.9, H-9); 10.31 (1H, d, ^3J = 7.9, CHO).

Ester (7*Z*)-**6a** was obtained as a crystalline compound; R_f 0.57 (1:2 EA–PE); mp 82–99°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.55 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.12 (3H, s, CH_3); 3.23 and 3.60 (2H, two d, AB system, ^2J = 28.2, SCH_2); 5.46 (1H, s, H-6); 6.55 (1H, d, ^3J = 6.4, H-9); 9.80 (1H, d, ^3J = 6.4, CHO).

***tert*-Butyl Ester of (7*E*)-(1-Formylethylidene)-3-methylceph-3-em-4-carboxylic acid ((7*E*)-6b)** and ***tert*-Butyl Ester of (7*Z*)-(1-Formylethylidene)-3-methylceph-3-em-4-carboxylic acid ((7*Z*)-6b).** Employing the procedure for the preparation of ester (7*Z*)-**4a**, ester **1** (174 mg, 0.647 mmol) in dichloromethane (25 ml) and 1-formylethylidene(triphenyl)phosphorane (**5b**) (206 mg, 0.647 mmol) gave a residue after evaporation of the reaction mixture. Column chromatography using 1:6 EA–PE as the eluent gave 195 mg (98%) of the (7*E*)- and (7*Z*)-isomers of ester **6b** mixture. Found, %: C 57.85; H 5.98; N 4.44. $C_{15}H_{19}NO_4S$. Calculated, %: C 58.23; H 6.19; N 4.53.

The isomers mixture obtained was separated by column chromatography using 1:20 EA–PE as the eluent. The fraction with R_f 0.49 (1:3 EA–PE) gave 70 mg (35%) (*E*)-isomer (7*E*)-**6b**; mp 146–150°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.56 (9H, s, C(C<u>H</u>$_3$)$_3$); 1.92 (3H, s, 3-CH_3); 2.09 (3H, s, 9-CH_3); 3.21 and 3.56 (2H, two d, AB system, ^2J = 18.3, SCH_2); 5.29 (1H, s, H-6); 10.36 (1H, s, CHO). ESI-MS mass spectrum: Found: *m/z* 332. $C_{15}H_{19}NO_4S$. Calculated : M = 309. $[M+Na]^+$ = 332.

The fraction with R_f 0.51 (1:3 EA–PE) gave 102 mg (51%) (*Z*)-isomer (7*Z*)-**6b**; mp 132–152°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.57 (9H, s, C(C<u>H</u>$_3$)$_3$); 2.10 (3H, s, 3-CH_3); 2.13 (3H, s, 9-CH_3); 3.21 and 3.59 (2H, two d, AB system, ^2J = 18.5, SCH_2); 5.41 (1H, s, H-6); 9.73 (1H, s, CHO). ESI-MS mass spectrum: Found: *m/z* 310. $C_{15}H_{19}NO_4S$. Calculated: M = 309, $[M+H]^+$ = 310.

***tert*-Butyl Ester of (6*Z*)-(N-Methoxycarbamoyl-N-methylmethylidene)-1,1-dioxopenicillanic Acid (9a).** Triphenylphosphorane **2a** (363 mg, 1.0 mmol) was added to a solution of *tert*-butyl ester of 6-oxopenicillanic acid (**7**) (271 mg, 1.0 mmol) in dichloromethane (20 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C and maintained for 16 h at –20°C. Then, the mixture was evaporated at reduced pressure. Column chromatography of the residue with 1:8 EA–PE as the eluent gave 350 mg (98%) ***tert*-butyl ester of (6*Z*)-(N-methoxycarbamoyl-N-methylmethylidene)penicillanic acid (8a)**; R_f 0.29 (1:2 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.50 (9H, s, C(C<u>H</u>$_3$)$_3$); 1.52 (3H, s, 2-CH$_3$); 1.57 (3H, s, 2-CH$_3$); 3.26 (3H, s, NCH$_3$); 3.73 (3H, s, OCH$_3$); 4.42 (1H, s, H-3); 6.04 (1H, s, H-5); 6.85 (1H, s, H-8).

According to the procedure for compound (7*Z*)-**4a**, a solution of the ester obtained **8a** (350 mg, 0.98 mmol) in dichloromethane (20 ml) was oxidized using 70% MCPBA (530 mg, 2.45 mmol) for 16 h. After work-up of the reaction mixture, column chromatography using 1:5 EA–PE as the eluent gave 201 mg (53%) penicillanic acid **9a**; R_f 0.29 (1:1 EA–PE); mp 133–135°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.47 (3H, s, 2-CH$_3$); 1.52 (9H, s, C(C<u>H</u>$_3$)$_3$); 1.59 (3H, s, 2-CH$_3$); 3.30 (3H, s, NCH$_3$); 3.76 (3H, s, OCH$_3$); 4.36 (1H, s, H-3); 5.56 (1H, s, H-5); 7.18 (1H, s, H-8). Found, %: C 49.63; H 6.16; N 7.05. $C_{16}H_{24}N_2O_7S$. Calculated, %: C 49.47; H 6.23; N 7.21.

***tert*-Butyl Ester of (6*Z*)-(3-Ethoxycarbonyl-2-hydroxyprop-2-enylidene)-1,1-dioxopenicillanic Acid (9b).** According to the procedure described for compound (7*Z*)-**4b**, the action of sodium hydroxide (40 mg) in water (5 ml) on 3-ethoxycarbonyl-2-oxopropylidene(triphenyl)phosphonium chloride (426 mg, 1.0 mmol) gave phosphorane **2b**, which, without purification, was dissolved in dichloromethane (10 ml). The resultant solution was added to ester **7** (271 mg, 1.0 mmol) in dichloromethane (20 ml). The reaction mixture was stirred for 2 h at 0°C, maintained for 16 h at –20°C, and evaporated at reduced pressure. Column chromatography using 1:10 EA–PE as the eluent gave 92 mg (24%) ***tert*-butyl ester of (6*Z*)-(3-ethoxycarbonyl-2-hydroxyprop-2-enylidene)penicillanic acid (8b)** as an amorphous substance with R_f 0.71 (1:2 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.29 (3H, t, *J* = 7.4, CH$_2$C<u>H</u>$_3$); 1.49 (3H, s, 2-CH$_3$); 1.50 (9H, s, C(CH$_3$)$_3$); 1.53 (3H, s, 2-CH$_3$); 4.23 (2H, q, *J* = 7.4, C<u>H</u>$_2$CH$_3$); 4.42 (1H, s, H-3); 5.29 (1H, s, H-5); 6.03 (1H, s, H-8); 6.27 (1H, s, H-10); 11.66 (1H, s, OH).

Employing the procedure for compound (7*Z*)-**4a**, resultant ester **8b** (91 mg, 0.237 mmol) was oxidized by MCPBA (102.4 mg, 0.593 mmol). After work-up of the reaction mixture, column chromatography using 1:5 EA–PE as the eluent gave 36.8 mg (37%) **9b**; R_f 0.50 (1:2 EA–PE); mp 136–140°C. ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.30 (3H, t, *J* = 7.4, CH$_2$C<u>H</u>$_3$); 1.46 (3H, s, 2-CH$_3$); 1.52 (9H, s, C(CH$_3$)$_3$); 1.58 (3H, s, 2-CH$_3$); 4.24 (2H, q, *J* = 7.4, C<u>H</u>$_2$CH$_3$); 4.34 (1H, s, H-3); 5.38 (1H, s, H-5); 5.46 (1H, s, H-8); 6.62 (1H, s, H-10); 11.69 (1H, s, OH). Found, %: C 52.18; H 6.25; N 3.37. $C_{18}H_{25}NO_8S$. Calculated, %: C 52.04; H 6.07; N 3.37.

(7*Z*)-(N-Methoxycarbamoyl-N-methylmethylidene)-3-methyl-1,1-dioxoceph-3-em-4-carboxylic Acid (10). Trifluoroacetic acid (1.5 ml) was added to a solution of ester **4a** (113.5 mg, 0.294 mmol) in dichloromethane (15 ml). The reaction mixture was stirred at room temperature for 24 h and evaporated under reduced pressure. Column chromatography of the residue using 3:1 EA–PE as the eluent gave 54.5 mg (56%) acid **10**, R_f 0.22 (3:1 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.96 (3H, s, CH$_3$); 3.19 (3H, s, NCH$_3$); 3.74 (3H, s, OCH$_3$); 4.16 and 4.31 (2H, two d, AB system, ^2J = 19.2, SO$_2$CH$_2$); 6.07 (1H, s, H-6); 7.17 (1H, s, H-9); 13.67 (1H, br. s, CO$_2$H). Found, %: C 43.38; H 4.25; N 8.39. $C_{12}H_{14}N_2O_7S$. Calculated, %: C 43.63; H 4.27; N 8.48.

(6*Z*)-(N-methoxycarbamoyl-N-methylmethylidene)-1,1-dioxopenicillanic Acid (11). Trifluoroacetic acid (1.5 ml) was added to a solution of ester **9a** (70 mg, 0.18 mmol) in dichloromethane (15 ml). The reaction mixture was stirred at room temperature for 120 h and evaporated at reduced pressure to give 53 mg (82%) of acid **11**; R_f 0.20 (3:1 EA–PE). ^{1}H NMR spectrum, δ, ppm (*J*, Hz): 1.45 (3H, s, 2-CH$_3$); 1.70 (3H, s, 2-CH$_3$); 3.21 (3H, s, NCH$_3$); 3.75 (3H, s, OCH$_3$); 5.08 (1H, s, H-3); 6.87 (1H, s, H-5); 7.31 (1H, s, H-8); 13.53 (1H, br. s, CO$_2$H). Found, %: C, 43.18; H, 4.65; N, 8.19. $C_{12}H_{16}N_2O_7S$. Calculated, %: C, 43.37; H, 4.85; N, 8.43.

Determination of Cytotoxic Activity of Alkylidene-substituted Cephems *in vitro*. The cytotoxic properties of the compounds synthesized relative to cultures of monolayer cancer and normal cells at *c* from

$2 \cdot 10^4$ to $5 \cdot 10^4$ cells/ml (human fibrosarcoma HT-1080, murine hepatoma MG-22A, murine embryonal fibroblasts 3T3) were determined in 96-well microtiter plates using CV, MTT, and NR staining according to standard procedures [10].

Generation of NO Radicals by Cells. The determination of the nitric oxide radical concentration in the Gries cell medium according to [11] for the alkylidene-substituted cephems was carried out in 96-well plastic panels (well volume 200 μl, concentration of the compound tested 50 μg/ml). The concentrations of the NO radicals (in nmol) in the culture medium with cells surviving after incubation in the presence of the tested compound over 72 h using the following formula for calculating the specific NO generating activity for the compounds (TG):

$$TG = G\ 100/C\ (\text{nmol}/\mu\text{l}),$$

where G is the NO concentration (nmol) in the culture medium (200 μl) with surviving cells and C is the percentage of surviving cells determined upon their CV staining.

Spectrophotometric Determination of the Inhibiting Properties of Alkylidene-substituted Cephems and Penams Relative to β-Lactamase. The total volume of the reaction mixture in each well of the 96-well plate was 200 μl and was obtained by adding 20 μl solution of *Bacillus cereus* β-lactamase (Sigma, P0389) (the final concentration in the reaction mixture was 0.8 E) or *Enterobacter cloacae* β-lactamase (Sigma, P4524) in 0.1 M phosphate buffer (pH 7.0) (the final concentration in the reaction mixture was 0.3 U). Then, 160 μl phosphate buffer (pH 7.0) containing the compound tested or Tazobactam in amounts sufficient to achieve concentrations of 0.2, 0.78, 3.13, 12.5, 50, and 200 μM was added. The mixture was incubated for 10 min and then 20 μl 0.97 μM (final concentration in the reaction mixture was 0.097 μM) solution of Nitrocefin in a 1:19 mixture of DMSO and 0.1 M phosphate buffer (pH 7.0) was added. The measurement of the optical density at 486 nm was carried out over 5 min and the result obtained was compared with the result of a control experiment, which was carried out without addition of the compound tested and Tazobactam to the solution of penicillinase and Nitrocefin. The IC_{50} values characterizing the concentration of the inhibitor reducing the activity of the enzyme by 50% was calculated using the Graph Pad Prism®.

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