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Manganese(III) promoted cyclization of *N*-alkenyl-*N*-(2-hydroxyethyl)amides to iso-oxacepham potent β-lactamase inhibitors

Paweł Punda[a], Marta Schielmann[b] and Sławomir Makowiec[*a]

[a]*Department of Organic Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12, 80 233 Gdańsk, Poland;*[b]*Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12, 80-233 Gdańsk, Poland*

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Abstract: Iso-oxacepham derivatives were synthesized by the 4-exo-trig radical cyclization as innovative one-pot approach. Subsequent cyclization process of *N*-alkenyl-(2-hydroxyethyl)amides to 7-substituted iso-oxacepham is described below including preparation of starting N-alkenylamides. Influence of carbamoyl, thiocarbamoyl and phosphoryl moieties located on C-7 position of iso-oxacephamic scaffold on β-lactamase inhibitory activity was confirmed on bacterial β-lactamases from group C.

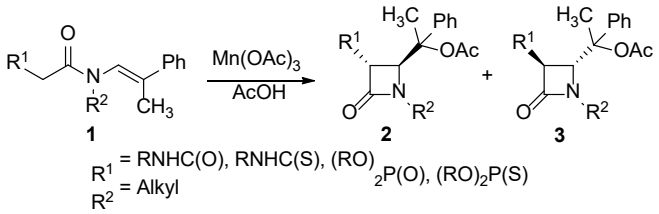
Keywords: β-lactams, β-lactamase inhibitors, iso-oxacephams, radical cyclizations, *N*-alkenylamides, cyclization.

1. INTRODUCTION

Through the decades, research aimed at the synthesis of new antibacterial agents have been focused mainly on the preparation of β-lactams. The increasing number of drug-resistant bacteria causes the necessity to seek for new modified structures. One approach to overcome bacterial resistance, is a modification of the core of antibiotics molecule. Classical penam/penem or cepham/cephem core could be replaced by oxa- or carba- analogs. Good examples of such a strategy are oxacephems antibiotics Flomoxef, and Latamoxef. Similarly, iso-oxacephems gained the attention due to their antibacterial activity [1, 2].

Taking into account the reports concerning carbamoyl β-lactams as inhibitors of bacterial β-lactamases [3], we focused our efforts on development of an easy route providing 7-substituted-iso-oxacephams. Previously we presented synthetic approach to form 3-carbamoyl-monobactams, based on modification of Staudinger ketene-imine cycloaddition [4].

Methods using 4-exo-trig radical cyclization leading to β-lactams are an alternative to classical Staudinger`s method [5]. Our research group has also made certain progress in this topic, as we developed method for preparation of *N*-alkyl/arylacarbamoyl-β-lactams **2**, **3** and their sulfur analogues based on radical cyclization [6]. Moreover, we applied $Mn(OAc)_3$ promoted radical cyclization to synthesis of difficult to achieve 3-phosphoryl and 3-thio phosphoryl-β-lactams (Scheme 1) [7].



Scheme 1. Synthesis of 3-substituted monobactams *via* radical cyclization of *N*-alkenylamides

Taking into account mechanistic aspects of the radical process, which is terminated by carbocation trapping by external nucleophile, we establish the hypothesis that introduction of internal nucleophile moiety into the substrate structure will allow to obtain 7-substituted-iso-oxacephams.

This approach would lead to formation of two heterocyclic rings in one-pot process. That is a significant advantage in comparison to synthesis of oxacephams presented so far in literature [8], where construction of oxacephamic scaffold is a result of more than one step.

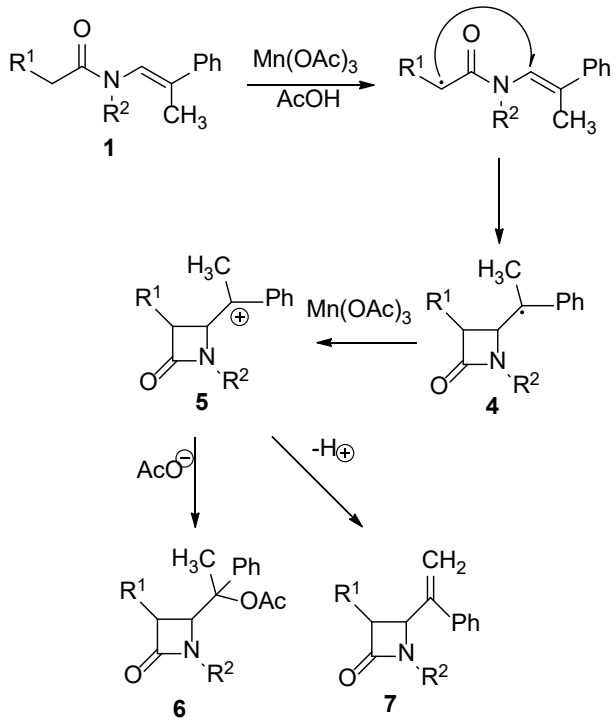
Based on the literature studies of the structure-antimicrobial activity relationship, we suspected low antibacterial activity of the final 7-substituted-iso-oxacephams [9, 10, 11]. However, our goal was to check β-lactamase inhibitory activity of 7-substituted-iso-oxacephams with carbamoyl, thiocarbomoyl or phosphoryl moieties.

In this paper, we would like to present our studies on synthesis of 7-substituted-iso-oxacephams and their biological evaluation as potential β-lactamases inhibitors.

2. RESULTS AND DISCUSSION

Chemistry

Snider and D`Annibale have proposed carbocation **5** formation as the last intermediate in the course of radical cyclization of 3-oxoenamides [5a, c, e, f] (Scheme 2). As one can easily predict introduction of an additional nucleophile will influent on products distribution. Surprisingly, due to strong coordination of manganese cation with acetoxy anions, during oxidation of the radical intermediate **4** with $Mn(OAc)_3$ we observed formation of the acetate **6** as the main product even if the experiment was performed in MeOH [6].

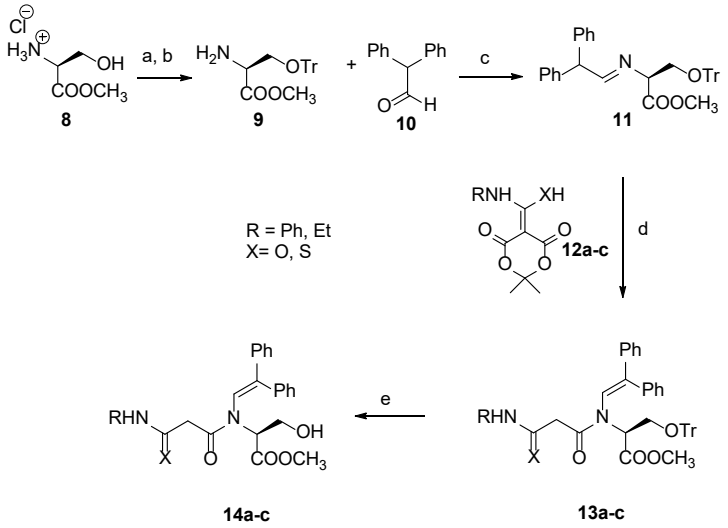


Scheme 2. Tentative mechanism for radical cyclization of N-alkenylamides to β-lactams.

Therefore, we took under investigation intramolecular process of carbocation - nucleophile reaction. We postulated that introduction of nucleophilic substituent into the *N*-alkenylamide structure like 2-hydroxyethyl side chain would provide bicyclic product of subsequent cyclization.

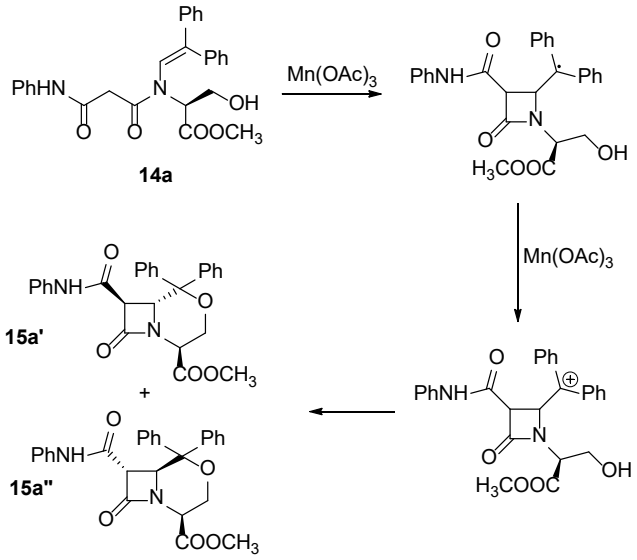
We prepared *N*-alkenyl-*N*-(2-hydroxyethyl)amides to consider our hypotheses with action of external nucleophile. For preliminary experiments, we chose enamides prepared from L-serine methyl ester as a starting material (Scheme 3).

Selective protection of hydroxyl group of **8** gave amine **9**, which treated with diphenylacetaldehyde **10** led to formation of enolizable imine **11**. Imine **11** was immediately acylated with ketene generated in thermal decomposition of Meldrum`s acid derivatives **12a-c**. The resulting products **13a-c** were deprotected by the treatment with solution of AcOH:TFE:TFA in DCM that gave better results than solution of TFA in DCM.



Scheme 3. Synthesis of *N*-alkenyl-*N*-(2-hydroxyethyl)amides. a) Tr-Cl, NEt_3; b: TFA; c: 4Å molecular sieves; d: reflux in toluene; e: AcOH, TFE, TFA.

Enamide **14a**, treated with $Mn(OAc)_3*2H_2O$ in acetic acid at 70°C gave expected 2-oxacephams **15a** in a first trial (Scheme 4).



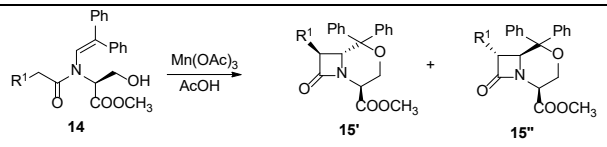
Scheme 4. Radical cyclization of *N*-alkenyl-*N*-(2 hydroxyethylene)amide to 7-substituted-O-2-isocepham. Absolute configuration on C-6 and C-7 was assigned arbitrary.

From the reaction mixture we isolated the pair of diastereoisomers **15a`** and **15a``** with 44% yield.

The first parameter that we tried to optimize was the temperature of the reaction. Temperatures below 70°C gave only trace of products, but the increase of temperature to boiling point of AcOH caused growth of the reaction rate and efficiency up to 55%. Reaction took only 10 sec and progress of the reaction was easy to observe by the disappearance of the brown color of the $Mn(AcO)_3$. The amount of oxidizer was established experimentally, $Mn(ACO)_3$ was added portionwise to a solution of **14a** in boiling AcOH until the consumption of new portion of oxidizer was observed. Fast disappearance of oxidizer ended up with 1.7 eq of $Mn(OAc)_3$ for 1 eq of **14a**. An independent experiment carried out with previously specified amount of oxidizer (1.7eq) in boiling AcOH gave 65% yield of **15**. We also checked the possibility of using CAN in this reaction instead of $Mn(OAc)_3$, but obtained yield was much lower.

To specify substrate spectrum of presented above method we tested representative group of *N*-alkenylamides bearing carbamoyl, thiocarbamoyl or phosphono moieties on amide α carbon **14a-d**. Although, in the case of *N*-alkenylphosphonamide we had to extend reaction time. All *N*-alkenyl-*N*-(2-hydroxyethyl)amides treated with $Mn(OAc)_3$ gave corresponding 7-substituted-iso-oxacephams **15'a-d, 15"a-d**. The obtained yields are presented in the Table 1.

Table 1. Synthesis of 7-substituted-iso-oxacephams.



Entry	**14**	R^1	Yield of (**15'**+ **15"**)	Ratio[a] **15'**: **15"**
1	**a**	PhNHC(O)	65	3:2
2	**b**	EtNHC(S)	40	3:2
3	**c**	EtNHC(O)	62	3:2
4	**d**	$(EtO)_2P(O)$	51	3:2

[a] Diasteroisomeric ratio calculated from ^{1}H NMR spectra
Absolute configuration arbitrary was assigned

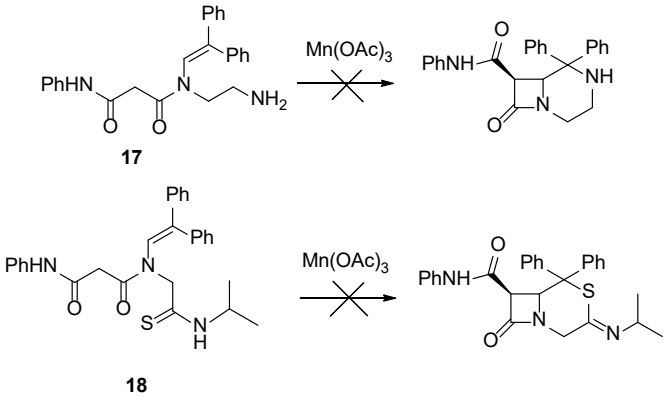
All obtained iso-oxacephams have trans configuration of protons HC(6) and HC(7), confirmed by the coupling constants in the range (2.0-2.5 Hz) on ^{1}H NMR spectra. Due to trans configuration of β-lactamic protons and configuration of asymmetric center 2(*S*), number of isomers formed in the reaction is limited to two. We tried to assign absolute configuration of asymmetric centers formed in the reaction relatively to the known 2(*S*) center by NOE NMR experiments. The assignment of absolute configuration was impossible because of the lack of significant differences between the diastereoisomers spectra.

Finally, obtained derivatives of iso-oxacephams were converted into the sodium salts **16** to provide solubility in water, during the biological assays.

Following our studies on the radical cyclization we decided to examine possibility to obtain isocephams and iso-azacephams using our strategy. We expected some side reactions, but to verify our hypothesis we prepared *N*-alkenylamides bearing N or S nucleophile in side chains that would react in analogical manner to hydroxyl group in synthesis of iso-oxacephams.

N-alkenylamides **17** and **18** were treated with $Mn(AcO)_3$ using standard conditions of radical cyclization reactions. Despite our best efforts we did not get desired products (Scheme 5).

In both cases we got inseparable mixture of side products of oxidation of substrates as well as other side reactions.



Scheme 5. Attempts to cyclization of N-alkenylamides bearing nitrogen and sulfur nucleophilic side chains.

Biological activity

Antimicrobial activity

All of the synthetized compounds have solely β-lactamase inhibitory activity. No antibacterial activity *in vitro* (up to the concentration of 1024 μg/mL) towards all of the tested reference strains and subclinical isolates of bacteria were noticed. As a reference compound, ampicillin was used. MIC values were as follows: 0.5 μg/mL for *Staphylococcus aureus* ATCC 29213, 10 μg/mL for *Staphylococcus aureus* PCM 2051, 2 μg/mL for *Staphylococcus epidermidis* PCM 2118, 8 μg/mL for *P. aeruginosa* ATCC 27853. *E. coli* ATCC 29425 was not sensitive to ampicillin up to the concentration of 1024 μg/mL.

β-lactamase inhibition

The β-lactamase inhibition was observed in case of four synthesized compounds: **16a`**, **16a``**, **16b`** and **16b``** (Table 2). For all of the synthetized compounds there were no enzymatic hydrolysis noticed (unpublished data). Our results showed that those compounds are not good inhibitors especially of the enzyme type A. Compounds **16a`**, **16a``**, **16b`** and **16b``** are inhibitors of enzyme C P99 at the concentration of 3.1 mM, 2.6 mM, 4.9 mM and 4.6 mM, respectively. Both enzymes, enzyme type A *B. cereus* and enzyme type C P99 were sensitive to tazobactam (TB), with IC_{50} values equal to 15 μM and 5 μM, respectively. Potassium clavulanate (CL) significantly reduced enzyme A activity (IC_{50}=0.069 mM), whereas showed no inhibitory potential up to 0.2 mM towards enzyme C (Table 2). As it was indicated, TB is a better inhibitor of β-lactamases than CL [12]. Inhibitory activity of TB was higher towards enzyme type C than type A, whereas Pauker and co-workers indicated that commercially available inhibitors such as TB and CL have rather better inhibitory properties against enzyme class A (TEM-1) than C [13]. This conclusion is true when compare CL and its effect on both enzymes.

Table 2. Inhibitory concentration[a] of synthetized and reference compounds towards enzyme type A (*Bacillus cereus I*) and enzyme type C (*Enterobacter cloacae*, P99).

Compound	IC_{50} [mM] (95% CI)	
	Enzyme A *B. cereus I*	Enzyme C P99
16a'	-	3.1 (2.8-3.4)
16a"	-	2.6 (2.5-2.9)
16b'	-	4.9 (4.6-5.2)
16b"	-	4.6 (4.3-4.9)
16c'	-	-
16c'	-	-
16d'	-	-
16d'	-	-
CL	0.069 (0.049-0.096)	-
TB	0.015 (0.011-0.019)	0.005 (0.004-0.006)

[a]Assay was performed independently, three times. '-' - no activity was observed for compounds **16a-d** up to 10 mM; in case of CL up to 0.2 mM. CL - potassium clavulanate; TB - tazobactam; CI - confidence intervals.

Synergy assay – combination disk methods

Based on enzyme inhibitory activity of four of the tested compounds **16a`**, **16a``**, **16b`** and **16b``**, assays regarding synergistic effects using disk diffusion method were performed. Stains identified as the β-lactamase producers *E. coli* ATCC 29425, *P. aeruginosa* ATCC 27853 and all subclinical *S. aureus* strains (SA83, SA84, SA103) were used in this assay. Although the amounts of tested compound on disk was 200 μg, there was no synergy observed with penicillin (10 μg), ampicillin (10 μg) or amoxicillin (20 μg). However, in the case of reference compounds, potassium clavulanate (10 μg) and tazobactam (10 μg), significant increase in the inhibition zones was noticed. Exemplary inhibition zones are shown in Fig. 1.

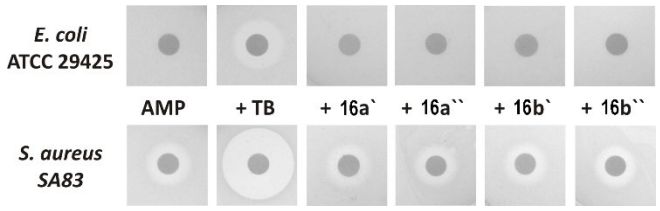


Figure 1. Synergic activity of reference and potent inhibitors against β-lactamase-positive strains. First disk contains ampicillin (AMP); next disks contain ampicillin and tazobactam (TB) or potent inhibitor.

3. CONCLUSION

Innovative synthetic approach to iso-oxacephams was confirmed. Closure of two heterocyclic rings step by step in one-pot occurs in accordance with our hypothesis. Representative group of iso-oxacephams showed various activity of inhibition serine β-lactamases group C, depending on the structure.

4. EXPERIMENTAL

Reagents and enzymes were purchased from Sigma-Aldrich. Nitrocefin was purchased from Merck. Toluene were distilled from potassium under argon. Dichloromethane was distilled from P_2O_5 under argon. Analytical TLC was performed on aluminum sheets of silica gel UV-254 Merck. Flash chromatography was performed using 40-63 microns of Zeochem silica gel. The ^1H, ^{13}C were recorded on Varian Gemini 200 and Varian Unity Plus 500, chemical shifts (δ) in ppm rel. to internal Me_4Si; coupling constants J in Hz. High-resolution (HRMS) was recorded on *MicroMas Quattro LCT* mass spectrometer. Melting points were determined with *Warsztat Elektromechaniczny W-wa* apparatus and were not corrected.

Procedure for the synthesis. Methyl (2*S*)-2-amino-3-(trityloxy)propanoate (**9**) was obtained in the reaction of hydrochloride of L-serine methyl ester with trifenylmethyl chloride in presence of triethylamine according to the method presented in literature by Rajca and co-workers [14]. ^1H NMR spectrum of the product (**9**) obtained was consistent with the literature. Methyl (2*S*)-2-[(2,2-diphenylethenyl)amino]-3-(triphenylmethoxy)propanoate (**11**) was obtained as described earlier [6, 15]. Freshly prepared imine was used in further step.

***N*-Alkenyl-*N*-(2-triphenylmethoxyethyl)amides (13a-c)** were prepared according to literature procedure [16].

(2S)-2-{(2,2-Diphenylethenyl)[3-oxo-3-(phenylamino)propanoyl]amino}-3-(triphenylmethoxy)propionic acid methyl ester(13a)

Purified by column chromatography in solvent system AcOEt/hexane 1:3 Yield = 60%. White foam. $[\alpha]_D^{22}$ = 33.9° (c=0.00383, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$) δ(ppm): 3.25 (1 H, d, ^2J = 17.7 Hz), 3.40 (1 H, d, ^2J = 17.7 Hz), 3.56 (2 H, d, ^3J = 12.0 Hz), 3.70 (3 H, s), 4.90 (1 H, m), 6.66 (1 H, s), 7.16-7.37 (30 H, m), 9.46 (1 H, s).

^{13}C NMR (50 MHz, $CDCl_3$) δ(ppm): 42.2, 52.5, 60.9, 61.1, 87.5, 120.0, 124.1, 124.2, 127.2, 127.8, 128.3, 128.5, 128.7, 128.8, 129.6, 136.8, 137.9, 139.6, 142.1, 143.0, 163.6, 169.1, 169.3.

HRMS (ESI): m/z $[M\text{-}CPh_3+H]^+$ calculated for $C_{27}H_{25}N_2O_5$: 459.1914, found: 459,1903.

(2S)-2-{(2,2-Diphenylethenyl)[3-(ethylamino)-3-oxopropanoyl]amino}-3-(triphenylmethoxy)propionic acid methyl ester (13b)

Purified by column chromatography in solvent system AcOEt/hexane 1:1 Yield = 37%. White foam. $[\alpha]_D^{22}$ = 69.6° (c =0.00416, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$) δ(ppm): 1.12 (3 H, t, ^3J = 7.3 Hz), 2.94 (1 H, d, ^2J = 17.4), 3.22-3.32 (3 H, m), 3.66 (3 H, s), 3.73-3.76 (2 H, m), 4.81-4.84 (1 H, m), 6,63 (1 H, s), 7.12-7.38 (26 H, m).

^{13}C NMR (50 MHz, $CDCl_3$) δ(ppm): 14.5, 34.3, 41.3, 52.3, 60.7, 61.3, 87.6, 118.2, 124.5, 127.2, 127.9, 128.2, 128.3, 128.3, 128.4, 128.6, 128.7, 129.7, 137.0, 139.8, 141.8, 143.1, 165.3, 169.4, 169.4.

HRMS (ESI): m/z $[M\text{-}CPh_3\text{-}H]^+$ calculated for $C_{23}H_{26}N_2O_5$: 409.1758; found: 409.1760.

(2S)-2-{(2,2-Diphenylethenyl)[3-(ethylamino)-3-thioxopropanoyl]amino}-3-(triphenylmethoxy)propionic acid methyl ester (13c)

Purified by column chromatography in solvent system AcOEt/hexane 1:1 Yield = 48%. White foam. $[\alpha]_D^{22}$ = 5.4° (c=0.00183, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$) δ (ppm): 1.26 (3 H, t, ^3J = 7.3 Hz), 3.52 (1 H, d, ^2J = 18.1 Hz), 3.63-3.7 (5 H, m), 3.71-3.80 (2 H, m), 3.89 (1 H, d, ^2J = 18.1 Hz), 4.71-4.74 (1 H, m), 6.65 (1 H, s), 7.14-7.35 (25 H, m), 9.45 (1 H, s).

^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm):12.9, 41.0, 48.8, 52.3, 61.0, 87.6, 124.3, 127.2, 127.9, 128.3, 128.4, 128.5, 128.6, 128.7, 129.8, 136.9, 139.7, 142.1, 143.2, 169.1, 169.6, 193.7.

HRMS (ESI): m/z $[M\text{-}CPh_3\text{-}H]^+$ calculated for $C_{23}H_{26}N_2O_4S$: 427.1686, found: 427.1691.

(2S)-2-{[(Diethoxyphosphoryl)acetyl](2,2-diphenylethenyl)amino}-3-(triphenylmethoxy)propionic acid methyl ester (13d)

Was obtained according to method presented in the literature [7]. Purified by column chromatography in solvent system AcOEt/hexane 3:2 Yield = 20%. White foam. $[\alpha]_D^{22}$ = 7.9° (c=0.00883, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.26-1.31 (6 H, m), 2.62 (1 H, dd, $^2J_{PH}$ = 22.5 Hz, $^3J_{HH}$ = 15.1 Hz), 3.1 (1 H, dd, $^2J_{PH}$ = 20.5 Hz, $^3J_{HH}$=15.1 Hz), 3.63 (3 H, s), 3.71-3.74 (1 H, m), 4,07-4,14 (4 H, m), 4.23-4.25 (1 H, m), 4.85-4.87 (1 H, m), 6.79 (1 H, s), 7.18-7.41 (25 H, m).

^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 16.6 (d, ^2J = 6.1 Hz), 29.9, 33.4, 34.5, 52.3, 60.4, 62.4 (d, ^3J=3.5 Hz), 62.7 (d, ^3J = 3.5 Hz), 87.7, 126.3, 126.8, 127.3, 127.4, 128.1, 128.1, 128.4, 128.5, 128.7, 128.9, 129.2, 129.3, 130.1, 137.4, 139.8, 140.6, 143.5, 169.1.

HRMS (ESI): m/z $[M\text{-}CPh_3\text{-}H]^+$ calculated for $C_{43}H_{44}NO_7P$: 718.2928, found: 718.2553.

General method of detritylation of *N*-alkenyl-*N*-(2-*O*-Tritylethylene)amides (13) to *N*-alkenyl-*N*-(2-hydroxylethylene)amides (14)

To a stirred *N*-alkenyl-*N*-(2-*O*-Tritylethylene)amide (**13)** (1 mmol) in DCM (10 mL) in room temperature was added sequentially 2,2,2-trifluoroethanol (2 mL), acetic acid (1 mL) and trifluoroacetic acid (1.3 mL). After complete conversion of starting material (monitored by TLC) reaction mixture was alcalized with sat. Na_2CO_3 and extracted with DCM (3 x 20 mL).

Combined organic fractions were dried with $MgSO_4$ and evaporated under reduced pressure. Product **14** was purified chromatographically and obtained as an oil.

(2S)-2-{(2,2-Diphenylethenyl)[3-oxo-3-(phenylamino)propanoyl]amino}-3-hydroxypropanoic acid methyl ester (14a)

Purified by column chromatography in solvent system AcOEt/hexane 1:1 Yield = 85%. Yellow oil. $[\alpha]_D^{22} = 36.9°$ (c=0.00216, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 3.53 (2 H, s), 3.66-3.74 (1 H, m), 3.75 (3 H, s), 3.95 (1 H, dd, ^2J = 12.0 Hz, ^3J = 4.8 Hz), 4.24 (1 H, t, 5.2), 6.69 (1 H, s), 7.24-7.58 (15 H, m), 9.36 (1 H, s).

^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 42.5, 52.6, 60.8, 65.0, 120.1, 124.4, 125.1, 128.2, 128.4, 128.8, 128.9, 129.5, 136.6, 137.6, 139.1, 141.6, 142.7, 156.5, 163.4, 169.6, 170.2, 172.1, 175.2, 195.4.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{27}H_{25}N_2O_5$: 459.1914; found: 459.1913.

(2S)-2-{(2,2-Diphenylethenyl)[3-(ethylamino)-3-oxopropanoyl]amino}-3-hydroxypropanoic acid methyl ester (14b)

Purified by column chromatography in solvent system AcOEt/hexane 5:1 Yield = 78%. Yellow oil. $[\alpha]_D^{22} = 36.4°$

(c=0.0055, $CHCl_3$). ^{1}H NMR (200 MHz, CDCl3) δ (ppm): 1.14 (3 H, t, ^3J = 7.3 Hz), 3.28-3.36 (4 H, m), 3.66-3.74 (4 H, m), 3.88 (1 H, dd, ^2J = 12,0 Hz, ^3J=4,8 Hz), 4.19 (1 H, dd, ^3J = 5.6 Hz, ^3J = 5.0 Hz), 6.67 (1 H, s), 7.13-7.15 (1 H, bs), 7.18-7.41 (10 H, m).

^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 14.5, 34.3, 41.3, 52.3, 60.7, 61.3, 87.6, 118.2, 124.5, 127.2, 127.9, 128.2, 128.3, 128.3, 128.4, 128.6, 128.7, 129.7, 137.0, 139.8, 141.8, 143.1, 165.3, 169.4, 169.4.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{26}N_2O_5$: 411.1914; found: 411.1914.

(2S)-2-{(2,2-Diphenylethenyl)[3-(ethylamino)-3-thioxopropanoyl]amino}-3-hydroxypropanoic acid methyl ester (14c)

Purified by column chromatography in solvent system AcOEt/hexane 1:1 Yield = 46%. Yellow oil. $[\alpha]_D^{22} = 0.0°$ (c=0.00233, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 1.25 (3 H, t, ^3J = 7.3 Hz), 3,0 (1 H, bs), 3.60-3.7 (5 H, m), 3.80-3.95 (4 H, m), 4.12-4.15 (1 H, m), 4.71-4.74 (1 H, m), 6.74 (1 H, s), 7.22-7.41 (10 H, m), 9.27 (1 H, s).

^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 12.9, 41.1, 49.3, 52.4, 61.9, 65.2, 125.5, 128.2, 128.5, 128.7, 128.8, 128.9, 129.7, 136.8, 139.1, 142.5, 169.5, 170.6, 193.4.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{26}N_2O_4S$: 427.1686; found: 427.1688.

(2S)-2-{[(Diethoxyphosphoryl)acetyl](2,2-diphenylethenyl)amino}-3-hydroxypropanoic acid methyl ester (14d)

Purified by column chromatography in solvent system AcOEt/hexane 5:1 Yield = 75%. Yellow oil. $[\alpha]_D^{22} = 11.2°$ (c=0.00533, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.30 (3 H, t, ^3J = 7.1 Hz), 1.31 (3 H, t, ^3J = 7.1 Hz), 2.76 (1 H, dd, $^2J_{PH}$ = 22.4 Hz, $^2J_{HH}$ = 14.1 Hz), 3.2 (1 H, dd, $^2J_{PH}$ = 21.2 Hz, $^2J_{HH}$ = 14.1 Hz), 3.69 (3 H, s), 3.94 (2 H, d, ^3J=6.3 Hz), 4.08-4.23 (5 H, m), 6.86 (1 H, s), 7.25-7.41 (10 H, m).

^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 16.3 (d, ^2J = 3.3 Hz), 33.7, 35.0, 52.0, 60.0, 62.7 (d, ^3J = 6.5 Hz), 63.2 (d, ^3J = 6.3 Hz), 64.6, 127.8, 128.3, 128.4, 128.8, 130.0, 137.1, 139.4, 140.1, 166.1, 166.2, 169.1.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{24}H_{30}NO_7P$: 476.1833; found: 476.1838.

General method for radical cyclization of *N*-alkenyl-*N*-(2-hydroxylethylene)amides (14) to 7-substituted-iso-oxacephams (15). To a mixture of *N*-alkenyl-*N*-(2-hydroxylethylene)amide (**14)** (1 mmol) in boiling acetic acid (10 mL), $Mn(AcO)_3$*2 H_2O (2 mmol) was added. After disappearance of brown color from oxidizer reaction mixture was cooled and poured on ice water (50 mL) and extracted with DCM (5 x 20mL). Combined organic fractions were dried with $MgSO_4$ and evaporated under reduced pressure.

(2S)-8-Oxo-5,5-diphenyl-7-(phenylcarbamoyl)-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylic acid methyl ester (15a', 15a")

Purified by column chromatography in solvent system AcOEt/hexan 2:5 Yield (**15a`**+**15a``**) = 65%.

First diasteroisomer (**15a'**). Yellow oil.:

$[\alpha]_D^{22} = 2.4°$ (c=0.00833, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 3.43 (1 H, d, ^3J = 2.0 Hz), 3.61-3.71 (4 H, m), 4.33 (1 H, dd, ^3J = 12.2 Hz, ^3J = 7.7 Hz), 4.76 (1 H, t, ^3J=9.0 Hz), 4.96 (1 H, d, ^3J = 2.0 Hz), 7.08-7.16 (1 H, m), 7.24-7.52 (12 H, m), 7.70-7.74 (3 H, m).

^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 52.6, 53.3, 55.3, 60.3, 60.7, 82.7, 120.5, 125.3, 126.5, 127.8, 128.4, 128.7, 129.2, 129.5, 137.6, 137.5, 142.7, 143.2, 163.4, 165.2, 168.8.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{27}H_{24}N_2O_5$: 457.1758; found: 457.1758.

Second diasteroisomer (**15a"**). Yellow oil.:

$[\alpha]_D^{22} = -75.0°$ (c=0.00133, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 3.43 (1 H, d, ^3J = 2.4 Hz), 3.88-3.97 (4 H, m), 4.26-4.32 (2 H, m), 4.78 (1 H, d, ^3J = 2.4 Hz), 7.08-7.15 (1 H, m), 7.25-7.52 (12 H, m), 7.80-7.90 (3 H, m).

^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 53.6, 55.5, 55.9, 60.1, 62.2, 84.8, 120.6, 125.3, 127.1, 127.8, 128.5, 128.7, 129.1, 129.2, 129.5, 137.4, 142.3, 143.6, 163.8, 164.1, 169.1.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{27}H_{24}N_2O_5$: 457.1758; found: 457.1759.

(2S)-7-(Ethylcarbamoyl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylic acid methyl ester (15b', 15b")

Purified by column chromatography in solvent system DCM/MeOH 80:1 Yield (**15b`**+**15b``**) = 62%.

First diasteroisomer (**15b'**). Yellow oil:

$[\alpha]_D^{22}$ = 120.0° (c=0.001, $CHCl_3$). ^{1}H NMR (200 MHz, aceton-d6) δ (ppm): 1.06 (3 H, t, ^3J = 7.3 Hz), 3.19-3.26 (2 H, m), 3.31 (1 H, d, ^3J = 2.0 Hz), 3.66 (3 H, s), 3.70 (1 H, dd, ^3J = 11.7 Hz, ^3J = 8.3 Hz), 4.38 (1 H, dd, ^3J = 11.7 Hz, ^3J = 7.8 Hz), 4.78 (1 H, t, ^3J = 8.3 Hz), 4.93 (1 H, d, ^3J = 2.0 Hz), 6.94 (1 H, bs), 7.25-7.38 (6 H, m), 7.43-7.47 (2 H, m), 7.60-7.62 (2 H, m).

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{24}N_2O_5$: 409.1758; found: 409.1757.

Second diasteroisomer (**15b"**). Yellow oil:

$[\alpha]_D^{22}$ = -150.0° (c=0.00166, $CHCl_3$). ^{1}H NMR (200 MHz, aceton-d6) δ (ppm): 1.07 (3 H, t, ^3J=7.3 Hz), 3.16 (1 H, d, ^3J = 2.4 Hz), 3.20-3.26 (2 H, m), 3.86 (3 H, s), 3.99 (1 H, dd, ^3J = 12.2 Hz, ^3J = 4.9 Hz), 4.22 (1 H, dd, ^3J = 12.6 Hz, ^3J = 2.0 Hz), 4.32-4.33 (1 H, m), 4.77 (1 H, d, ^3J = 2.4 Hz), 6.96 (1 H, bs), 7.25-7.32 (3 H, m), 7.37-7.40 (3 H, m), 7.46-7.49 (2 H, m), 7.69-7.71 (2 H, m).

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{24}N_2O_5$: 409.1758; found: 409.1757.

(2S)-7-(Ethylcarbamothioyl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylic acid methyl ester (15c`, 15c")

Purified by „flash" column chromatography in solvent system AcOEt/hexan 1:1 Yield (**15c`**+**15c``**) = 40%.

First diasteroisomer (**15c'**). Yellow oil:

$[\alpha]_D^{22}$ = 46.1° (c=0.00216, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.24 (3 H, t, ^3J = 7.8 Hz), 3.43 (1 H, s), 3.56-3.69 (4 H, m), 3.70 (3 H, s), 4.29 (1 H, dd, ^3J = 12.2 Hz, ^3J = 7.8 Hz), 4.72 (1 H, dd, ^3J = 9.8 Hz, ^3J = 7.8 Hz), 5.12 (1 H, d, ^3J = 1.9 Hz), 7.18-7.45 (8 H, m), 7.80 (1 H, bs), 7.89 (2 H, d, ^3J = 7.3 Hz).

^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 12.9, 29.7, 40.6, 51.8, 52.7, 58.2, 59.3, 65.8, 82.7, 126.0, 127.5, 127.7, 128.0, 128.4, 128.5, 142.4, 142.9, 165.6, 168.3, 193.8.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{24}N_2O_4S$: 423.1373; found: 423.1385.

Second diasteroisomer (**15c"**). Yellow oil:

$[\alpha]_D^{22}$ = -75.0° (c=0.00066, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.19 (3 H, t, ^3J = 7.3 Hz), 3.48 (1 H, d, ^3J = 2.4 Hz), 3.60-3.65 (2 H, m), 3.87 (1 H, s), 3.98 (1 H, dd, ^3J = 12.7 Hz, ^3J = 4.9 Hz), 4.21 (1 H, dd, ^3J = 12.7 Hz, ^3J = 1.9 Hz), 4.33-4.35 (1 H, m), 5.22 (1 H, d, ^3J = 2.4 Hz), 7.24-7.48 (8 H, m), 7.75 (2 H, d, ^3J = 7.8 Hz), 8.97 (1 H, bs).

^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 12.9, 40.5, 51.8, 52.7, 58.2, 59.3, 65.9, 82.7, 126.0, 127.5, 127.7, 128.0, 128.4, 128.5, 142.4, 142.9, 165.6, 168.3, 175.2, 193.8.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{24}N_2O_4S$: 423.1373; found: 423.1381.

(2S)-7-(Diethoxyphosphoryl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (15d`, 15d")** Purified by column chromatography in solvent system AcOEt/hexane 2:1 Yield (**15d`+**15d``**) = 51%.

First diasteroisomer (**15d'**). Yellow oil:

$[\alpha]_D^{22}$ = 108.0° (c=0.00083, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.32 (3 H, t, ^3J = 7.3 Hz), 1.38 (3 H, t, ^3J = 6.8 Hz), 3.18 (1 H, dd, $^2J_{PH}$ = 16.1 Hz, ^3J = 2.0 Hz), 3.65 (1 H, dd, ^3J = 12.2 Hz, ^3J = 9.3 Hz), 3.70 (3 H, s), 4.15 (2 H, m), 4.19 (2 H, m), 4.29 (1 H, dd, ^3J = 12.2 Hz, ^3J = 7.3 Hz), 4.66 (1 H, dd, $^3J_{PH}$ = 5.9 Hz, ^3J = 1.9 Hz), 4.75 (1 H, t, ^3J=8.3 Hz), 7.23-7.33 (5 H, m), 7.34-7.36 (1 H, m), 7.42 (2 H, t, ^3J = 7.8 Hz), 7.57 (2 H, d, ^3J = 7.8 Hz).

^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 16.5, 16.6, 16.6, 29.9, 52.0, 52.9, 53.1, 53.4, 54.2, 60.0, 63.1, 63.2, 63.2, 82.6, 126.5, 127.4, 128.2, 128.5, 128.8, 128.9, 142.3, 142.4, 162.5, 168.8.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{24}H_{28}NO_7P$: 476.1676; found: 476.1676.

Second diasteroisomer (**15d"**). Yellow oil:

$[\alpha]_D^{22}$ = -116.0° (c=0.0025, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.28-1.34 (6 H, m), 3.03 (1 H, dd, $^2J_{PH}$ = 15.6 Hz, ^3J = 2.4 Hz), 3.90 (3 H, s), 3.98-4.20 (5 H, m), 4.26 (1 H, dd, ^3J = 12.2 Hz, ^3J = 2.4 Hz), 4.62 (1 H, dd, $^2J_{PH}$ = 7.8 Hz, ^3J = 2.4 Hz), 7.22-7.37 (6 H, m), 7.43 (2 H, t, ^3J=7.3 Hz), 7.60 (2 H, d, ^3J=7.8 Hz).

^{13}C NMR (200 MHz, $CDCl_3$) δ (ppm): 16.6 (t, ^3J = 7.0 Hz), 22.4, 53.1, 53.5, 53.6, 54.7, 61.9, 62.7 (d, ^2J = 6.1), 63.4(t, ^2J = 6.1), 83.7, 126.8, 127.6, 128.2, 128.5, 128.7, 128.9, 142.0, 142.9, 161.2, 161.2, 168.7.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{24}H_{28}NO_7P$: 476.1676; found: 476.1676.

General method for hydrolysis of methyl esters of 7-substituted-iso-oxacephams (15) to sodium salts (16). To a stirred solution of 7-substituted-*O*-2-isocepham **(15)** (1 mmol) in absolute ethanol (10 mL) 1M standard solution of NaOH (1 mL) was

added. After complete conversion of substrate mixture was evaporated. Final products **16** was obtained as white foams and used for biological assays.

Sodium (2S)-8-oxo-5,5-diphenyl-7-(phenylcarbamoyl) -4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16a')

$[\alpha]_D^{22}$ = 0.0° (c=0.00133, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 3.28 (1 H, s), 3.40 (1 H, t, ^3J = 11.7 Hz), 4.07 (1 H, t, ^3J = 8.3 Hz), 4.45 (1 H, t, ^3J = 8.3 Hz), 4.77 (1 H, s), 6.95-7.22 (15 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 54.1, 54.3, 60.7, 81.6, 121.7, 125.8, 127.2, 128.2, 128.5, 128.8, 128.9, 129.3, 136.3, 141.5, 142.9, 165.3, 166.2.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{26}H_{21}N_2NaO_5$: 465.1421; found: 465.1427.

Sodium (2S)-8-oxo-5,5-diphenyl-7-(phenylcarbamoyl) -4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16a``)

$[\alpha]_D^{22}$ = -15.0° (c=0.00133, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 3.26 (1 H, s), 3.42-3-50 (1 H, m), 4.05 (1 H, t, ^3J = 8.3 Hz), 4.45 (1 H, t, ^3J = 8.3 Hz), 4.80 (1 H, s), 6.99-7.25 (15 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 22.2, 45.4, 54.1, 54.3, 81.6, 121.9, 125.8, 126.1, 127.2, 128.6, 128.9, 128.9, 129.0, 129.3, 136.2, 142.9, 164.0, 174.7.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{26}H_{21}N_2NaO_5$: 465.1421; found: 465.1396.

Sodium (2S)-7-(ethylcarbamothioyl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16b`)

$[\alpha]_D^{22}$ = 23.08° (c=0.00216, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 0.93 (3 H, t, ^3J = 7.3 Hz), 3.28-3.34 (1 H, m), 3.36-3.43 (2 H, m), 3.51 (1 H, s), 4.07-4.11 (1 H, m), 4.33 (1 H, t, ^3J = 8.3 Hz), 5.05 (1 H, s), 7.00-7.17 (9 H, m), 7.31-7.33 (2 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 12.0, 41.4, 53.9, 57.5, 60.8, 65.9, 81.5, 125.6, 127.6, 128.1, 128.6, 129.0, 129.1, 141.3, 143.4, 167.7, 175.0, 192.6.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{22}H_{21}N_2NaO_4S$: 433.1192; found: 433.1195.

Sodium (2S)-7-(ethylcarbamothioyl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16b``)

$[\alpha]_D^{22}$ = -27.27° (c=0.00183, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 0.94 (3 H, t, ^3J = 7.3 Hz), 3.29-3.42 (3 H, m), 3.52 (1 H, s), 4.10-4.12 (1 H, m), 4.44 (1 H, t, ^3J = 8.3 Hz), 5.06 (1 H, s), 7.03-7.34 (11 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 12.0 41.4, 53.8, 57.5, 60.8, 66.0, 81.5, 125.6, 127.0, 127.6, 128.2, 128.7, 129.0, 129.1, 141.3, 143.4, 167.8, 175.0, 192.7.

HRMS (ESI): m/z $[M + 2H]^+$ calculated for $C_{22}H_{21}N_2NaO_4S$: 433.1192; found: 434.1196.

Sodium (2S)-7-(ethylcarbamoyl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16c`)

$[\alpha]_D^{22}$ = 96.0° (c=0.00166, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 0.90 (3 H, t, ^3J = 7.3 Hz), 2.98-3.09 (2 H, m), 3.17 (1 H, s), 3.51 (1 H, dd, ^3J = 11.7 Hz, ^3J = 8.8 Hz), 4.13 (1 H, dd, ^3J = 12.2 Hz, ^3J = 8.3 Hz), 4.47 (1 H, t, ^3J = 8.3 Hz), 4.75 (1 H, s), 7.09 (2 H, d, ^3J = 7.3 Hz), 7.19-7.33 (8 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 13.6 35.0, 54.3, 54.4, 54.5, 59.7, 60.9, 81.8, 126.0, 127.3, 128.4, 128.7, 129.0, 129.2, 141.6, 143.0, 167.0, 174.8.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{22}H_{21}N_2NaO_5$: 417.1421; found: 417.1418.

Sodium (2S)-7-(ethylcarbamoyl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16c``)

$[\alpha]_D^{22}$ = -48.0° (c=0.00166, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 0.90 (3 H, t, ^3J = 7.3 Hz), 2.98-3.09 (2 H, m), 3.16 (1 H, d, ^3J = 1.5 Hz), 3.50 (1 H, dd, ^3J = 12.2 Hz, ^3J=9.3 Hz), 4.13 (1 H, dd, ^3J = 11.7 Hz, ^3J = 7.8 Hz), 4.47 (1 H, t, ^3J = 8.3 Hz), 4.75 (1 H, d, ^3J = 2.9 Hz), 7.08 (2 H, d, ^3J = 6.8 Hz), 7.17-7.36 (8 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 13.6, 35.0, 39.0, 54.3, 54.4, 60.9, 81.8, 126.0, 127.0, 127.3, 128.4, 128.7, 129.0, 129.2, 141.6, 142.9, 166.7, 174.8.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{22}H_{21}N_2NaO_5$: 417.1421; found: 417.1425.

Sodium (2S)-7-(diethoxyphosphoryl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16d`)

$[\alpha]_D^{22}$ = 65.45° (c=0.00183, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 1.12-1.18 (6 H, m), 2.90 (1 H, d, $^2J_{PH}$=16.1 Hz), 3.55-3.57 (1 H, m), 3.94-4.03 (5 H, m), 4.15 (1 H, t, ^3J = 8.3 Hz), 4.50 (1 H, t, $^3J_{PH}$ = 7.8 Hz), 7.10 (2 H, d, ^3J = 7.3 Hz), 7.24-7.38 (8 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 15.8, 51.6, 53.0 (d, $^2J_{PC}$ = 8.8 Hz), 55.1, 61.0, 64.8 (d, $^2J_{PC}$ = 7.0 Hz), 64.9 (d, $^2J_{PC}$ = 7.0 Hz), 82.6, 126.5, 127.3, 128.8, 128.8, 129.0, 129.2, 141.6, 142.2, 174.4.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{25}NNaO_7P$: 482.1339; found: 482.1373.

Sodium (2S)-7-(diethoxyphosphoryl)-8-oxo-5,5-diphenyl-4-oxa-1-azabicyclo[4.2.0]octane-2-carboxylate (16d``)

$[\alpha]_D^{22}$ = -45.0° (c=0.00133, MeOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 1.12-1.17 (6 H, m), 2.90 (1 H, d, $^2J_{PH}$ = 16.6 Hz), 3.52-3.56 (1 H, m), 3.92-4.08 (5 H, m), 4.15 (1 H, dd, ^3J = 12.2 Hz, ^3J = 7.3 Hz), 4.50 (1 H, t, $^3J_{PH}$=7.8 Hz), 7.09-7.38 (10 H, m).

^{13}C NMR (200MHz, D_2O) δ (ppm): 15.7, 30.4, 52.9, 55.0, 60.9, 64.7 (d, $^2J_{P-C}$ = 6.6 Hz), 64.8 (d, $^2J_{PC}$ = 6.6 Hz), 82.5, 126.3, 126.7, 127.2, 128.7, 128.7, 128.9, 129.1, 141.5, 142.0, 164.0, 174.3.

HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{23}H_{25}NNaO_7P$: 482.1339; found: 482.1386.

Biological assays

Antimicrobial activity. The microbial activity of compounds was determined on set of reference bacterial Gram-positive strains: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* PCM 2051, *Staphylococcus epidermidis* PCM 2118 and Gram-negative: *Escherichia coli* ATCC 29425, *Pseudomonas aeruginosa* ATCC 27853. Ampicillin was used as a reference compound. The microbial activity was described as MIC (minimal inhibitory concentration) values and was evaluated using the broth microdilution method on 96-wells polystyrene plate according to the standard protocol of the CLSI (Clinical and Laboratory Standards Institute). Briefly, in these assays, final dilution of bacteria was 5 x 10^5 CFU/mL, the highest final concentration of tested compounds were 1024 μg/mL, the Mueller-Hinton II Broth (cation adjusted) was used as a growing medium. Inoculated microtiter plates were incubated at 35°C for 18 hrs. The MIC was defined as the lowest concentration of compound at which there was no visible growth.

Enzyme inhibition IC_{50}. The β-lactamase inhibition activity was evaluated using nitrocefin as chromogenic substrate as it was described previously by O'Callaghan [17] and Cierpucha [18] with slight modifications. Inhibition of nitrocefin hydrolysis was determined spectrophotometrically at 25°C for 10 min after 30 min pre-incubation of enzyme with compound dilutions, in the presence of 0.1 mM nitrocefin and 70 nM enzyme type A from *Bacillus cereus* or 68 nM enzyme type C from *Enterobacter cloacae* (P99) in a final volume of 0.125 mL. Assay was performed in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mg/mL of bovine serum albumin for enzyme stabilization [19]. The change in the absorbance at 490 nm was continuously read at 25°C for 10 min by microtiter plate reader Victor^3 (Perkin Elmer), using 96-well plates. Concentration of the β-lactamase enzymes was chosen due to obtain linear hydrolysis of nitrocefin for further than 5 min. The IC_{50} was defined as the inhibitory concentration that inhibits the enzyme activity in 50% in comparison to the control – enzyme with no inhibitor. Data was processed using nonlinear regression and dose response analysis with GraphPad Prism Version 5.01 (GraphPad Software, San Diego California USA) and expressed in mM with 95% confidence intervals.

Synergy assay – combination disk methods. Disc diffusion method were performed according to the guidelines of the CLSI. The β-lactamase positive strains were only used in this assay. Three *Staphylococcus aureus* isolates from bovine mastitis (coded as SA83, SA84, SA103) [20] were also tested. The β-lactamase production of tested strains was checked by direct method. The aliquots of 5 μl of nitrocefin solution were added on the surface of colony. The change of color from yellow to red informed about enzyme production [17]. The pour plate method were employed in this assay. An aliquot of 200 μl of bacteria dilution with cell density around 1.5 x 10^8 CFU/mL were added to warm 20 mL Mueller-Hinton II Agar (cation adjusted). Disks containing antibiotic (penicillin – 10 μg, ampicillin – 10 μg, amoxicillin – 20 μg) and inhibitor (tested compounds – 200 μg, reference inhibitors potassium clavulanate or tazobactam – 10 μg) were placed on the congealed plate surface. Plates were incubated at 35°C for 18 hr. Following the incubation, the diameters of the inhibition zone of discs were compared. The increase in the zone of inhibition around disk containing antibiotics and inhibitor indicates synergy between antibiotic and β-lactamase inhibitor.

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