Short communication

Synthesis of C6-Ethylidene Meropenem Derivative with Antimicrobial Activity

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Dedicated to Professor Branko Stanovnik on the occasion of his 70th birthday

Abstract

The development of novel β-lactamase inhibitors with activity against various clinically relevant β-lactamase producing strains is one of the most important strategies to sustain the clinical efficacy of β-lactam antibiotics. With intention to eliminate antibiotic activity of meropenem with preserved activity against β-lactamases C6-hydroxyethyl side chain of meropenem was transformed to C6-ethylidene moiety. IC50 values of C6-ethylidene derivative of meropenem were in low mM range against TEM-1, SHV-1 and AmpC enzymes and were clearly inferior to meropenem. Surprisingly, some of the antimicrobial activity of meropenem was preserved implying that C6-hydroxyethyl side chain is not essential to retain antibiotic activity of meropenem.

Keywords: Tricyclic carbapenem, antimicrobial, β-lactamase inhibitor

1. Introduction

The dramatic increase in antibacterial resistance in both community and hospital settings have resulted in limited effectiveness of current drugs and represent a real public health threat. A wide variety of pathogens have acquired antimicrobial resistances to β-lactam antibiotics by the synthesis of β-lactamases. Based on amino-acid sequence similarities, β-lactamases have been broadly grouped into four molecular classes, A, B, C and D. The β-lactamase superfamily currently has more than 700 members, many of which differ only by a single amino acid. Commercially available inhibitors potassium clavulanate, sulbactam and tazobactam, inhibit most class A and some class D β-lactamases, but activity against class C types is poor in general. NXL-104 is a non-β-lactam inhibitor currently in clinical phase I and there are several compounds under investigation as potential β-lactamase inhibitors.[1,2] However, there is a great unmet medical need for broad-spectrum inhibitor of β-lactamases, that would be capable to restore diminished activity of β-lactam antibiotic in combination and extend its antimicrobial spectrum.

Recently a development of novel broad-spectrum inhibitor of β-lactamases LK-157 has been published.[3,4] It has been shown that transformation of C10-hydroxyethyl side chain of sanfetrinem to C10-ethylidene moiety of LK-157 resulted in loss of antibiotic activity. The common feature of all carbapenems on the market is the C6-hydroxyethyl side chain which is believed to contribute to antibacterial activity and stability against β-lactamases. Therefore our initial assumption was to apply the same strategy also in case of meropenem to retain only β-lactamase inhibitory activity. In this paper, the synthesis of C6-ethyliden derivative of meropenem, its IC50s against TEM-1, SHV-1 and AmpC enzymes and antimicrobial activity are presented.

2. Results and Discussion

2. 1. Chemistry

Commercially available meropenem (**1**) was chosen as the starting compound for the synthesis of C6-ethylidene meropenem derivative LK-175 (**5**). The synthesis of C6-ethylidene analogue has already been reported, although the final compound has never been evaluated as inhibitor of β-lactamases.[5] In addition, the coupling of 3-ethylideneazetidinone fragment and 4-mercapto pyrrolidine carboxamide comprised of total 8 synthetic steps. The-

refore, we designed a shorter and more convenient synthetic route to the LK-175 (**5**). Carboxylate and amine moieties of meropenem were protected as allyl ester and allyl carbamate protecting groups, respectively, followed by water elimination resulting in formation of the ethylidene moiety. The final compound was isolated upon deprotection of allyl groups (Scheme 1).

Meronem®, commercially available mixture of meropenem (**1**) and sodium carbonate, was first treated with allyl chloroformate in water and additional quantity of sodium carbonate to afford the corresponding *N*-protected allyloxycarbonyl derivative **2**.[6] Water was removed by liophilisation, and the mixture alkylated with allyl bromide in DMF at room temperature.[7] The obtained allyl ester **3** was purified by extraction followed by column chromatography. Subsequently, water elimination under the conditions of the Mitsunobu reaction[8] afforded ethylidene derivative **4**, which was further purified by column chromatography. Both protecting groups were removed by tributyltin hydride in the presence of bis(triphenylphosphine)palladium(II) dichloride and water in dichloromethane.[9] The stannic by-products were removed by washing the crude product with diethyl ether, followed by preparative HPLC purification and liophilization to afford LK-175 (**5**).

2. 2. Biological Activity

The results of the inhibitory activities against class A (TEM-1 and SHV-1 from *Escherichia coli*) and class C (AmpC from *E. cloacae*) β-lactamases for LK-175, meropenem and tazobactam are presented in Table 1.

Table 1: *In vitro* activity of C6-ethylidene derivative of meropenem against class A (TEM-1 and SHV-1) and class C (AmpC) β-lactamases.

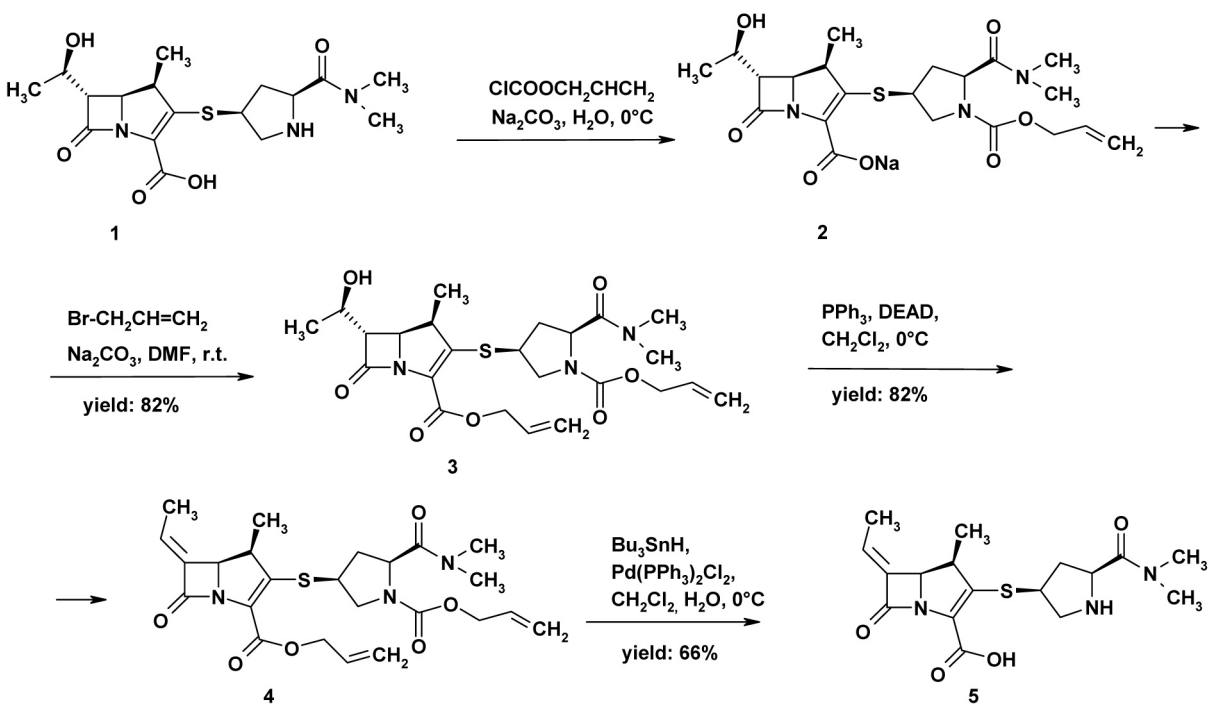
Compound	IC_{50} [nM] TEM-1	SHV-1	AmpC
Tazobactam	17	222	1808
Meropenem	200	200	1
LK-175	1000	2500	5000

IC50 values of LK-175 were in low mM range and were clearly inferior to meropenem, thus neglecting our hypothesis to prepare the equipotent inhibitor of β-lactamases by subtle structural modification.

Moreover, LK-175 exhibited antimicrobial profile against various bacterial strains as shown in Table 2. MIC values were in range of 1 to 4 μg/ml, except for MRSA-producing *S. aureus* ATCC 43300 with higher MIC values (8–16 μg/ml). Surprisingly, some of the antimicrobial activity of meropenem was preserved, although it is believed that C6-hydroxyethyl side chain is crucial for antibiotic activity of carbapenems and is a common feature of all carbapenem antibiotics on the market. However, our finding implies that C6-hydroxyethyl side chain is not vital to retain antibiotic activity.

3. Conclusions

There is no broad-spectrum inhibitor of β-lactamases on the market that covers also class C β-lactamases. A



Scheme 1. Synthetic route to LK-175 (5).

Table 2: MIC values [μg/ml] of LK-175 against a mini-panel of strains.

MIC (μg/ml)	S1	S2	S3	S4	S5	S6	S7	S8
LK-175	4/4	4/2	2/1	16/8	1/1	1/1	4/4	2/1

design of meropenem derivative LK-175 with C6-hydroxyethyl moiety was based on results of broad-spectrum β-lactamase inhibitor LK-157 encouraged by finding that meropenem is also a good inactivator of AmpC. However, LK-175 exhibited only a weak β-lactamase inhibitory activity. On the other side, it is interesting to note that C6-hydroxyethyl side chain is not essential to retain antibiotic activity and various modifications could be employed in future design of novel carbapenem antibiotics.

4. Experimental

4. 1. Synthesis

4. 1. 1. General Chemical Methods

Analytical TLC was performed on Merck silica gel 60 F_{254} plates (0.25 mm) and the components detected using specific spray reagents. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). DSC spectrum was recorded on a Mettler Toledo DSC822e. IR spectrum was obtained with a Nicolet FT-IR Nexus spectrometer and optical rotation was measured on a Perkin Elmer 1241 MC polarimeter. ^{1}H-NMR spectra were recorded on a Varian Inova300 spectrometer in DMSO-d_6 solution, with TMS as the internal standard. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. LC-MS analyses were performed on an Alliance HT Waters 2795 separations module with a Waters 2996 (photodiode array) UV-detector and a Micromass Quatro Micro mass spectrometer. HRMS analyses were performed on Micromass QTOF Ultima Global using ESI (negative and positive mode). HPLC analyses were carried out with Waters 2695 Separation Module with a Waters 2996 PDA detector and an XTerra RP C18 (150 × 4.6 mm I.D., 3.5 μm particle size) analytical column and a gradient elution method combining mobile phase A with 25 mM ammonium acetate (pH = 6) / acetonitrile (95/5 v/v) and mobile phase B with 25 mM ammonium acetate (pH = 6) / acetonitrile (10/90 v/v). All reported yields are yields of purified products.

Synthesis of (4*R*,5*S*,6*S*)-allyl 3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-[(*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (3). A commercially available product Meronem® (meropenem (**1**) + Na_2CO_3) (1g) was dissolved in distilled water (40 mL). Na_2CO_3 (0.300 g) was added and the mixture cooled to 0 °C, followed with addition of allyl chloroformate (0.30 mL, 0.341 g), stirred at 0 °C for 1 hour and then lyophilized (T = –20 °C, p = 0.05–0.08 mbar, overnight) to afford 1.866 g of a solid mixture of Na_2CO_3 and sodium (4*R*,5*S*,6*S*)-3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-[(R)-1-hydroxyethyl]-4-methyl-7-oxo-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylate (**2**). The obtained product was dissolved in DMF (30 mL) and Na_2CO_3 (0.558 g) and allyl bromide (0.25 mL, 0.317 g) were added and the reaction mixture additionally stirred for 2–3 days at room temperature. If necessary, additional quantity of allyl bromide was added (0.05 mL + 0.1 mL). After the complete conversion was detected by HPLC, the reaction mixture was filtered and the solvent removed under reduced pressure. The crude product was dissolved in ethyl acetate (80 ml) and washed with water (3 × 80 ml) to afford 1.08 g of (4*R*,5*S*,6*S*)-allyl 3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(methylcarbamoyl)pyrrolidin-3-ylthio]-6-[(*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylate (**3**); yield: 82%, yellow oil; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15 (d, 6H, *J* = 5.4 Hz), 1.52–1.64 (m, 1H), 1.95 (s, 1H), 2.76–2.86 (m, 1H), 2.97 (s, 3H), 2.99 (s, 3H), 3.02–3.11 (m, 1H), 3.21 (dd, 1H, J_1 = 4.5 Hz, J_2 = 2.1 Hz), 3.52–3.57 (m, 1H), 3.71–3.84 (m, 1H), 3.91–4.02 (m, 1H), 4.04–4.10 (m, 1H), 4.17–4.21 (m, 1H), 4.42–4.50 (m, 2H), 4.57–4.59 (m, 1H), 4.66–4.74 (m, 2H), 5.05 (d, 1H, *J* = 3.3 Hz), 5.14–5.19 (m, 2H), 5.35–5.40 (m, 2H), 5.83–5.93 (m, 2H) [peaks for H2'alfa and H6 are under signal for H_2O]. ^{13}C NMR (100 MHz, DMSO-d_6) δ 17.2, 21.9, 35.5, 35.6, 36.6, 43.1, 55.6, 56.2, 59.8, 60.0, 64.4, 65.0, 65.3, 116.4, 117.4, 117.9, 132.4, 133.4, 149.7, 153.2, 160.2, 170.7, 174.2. HRMS–FAB (*m/z*): calcd for $C_{24}H_{34}N_3O_7S$ (M+H), 508.2117; found, 508.2093. LCMS calcd for $C_{24}C_{33}N_3O_7S$: 507; obtd. 508 (M+H). HPLC: 93.3 area %.

Synthesis of (4*R*,5*R*,E)-allyl 3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-ethylidene-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (4). (4*R*,5*S*,6*S*)-allyl 3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(methylcarbamoyl)pyrrolidin-3-ylthio]-6-((*R*)-1-hydroxyethyl)-4-methyl-7-oxo-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylate (**3**) (3.15 mmol, 1.60 g) was dissolved in dry dichloromethane (20 mL) under argon atmosphere. Triphenylphosphine (1.05 EQ, 3.31 mmol, 0.869 g) and diethyl azodicarboxylate (1.05 EQ, 3.31 mmol, 0.577 g, 0.515 mL) were added during stirring on an ice-cooled bath. The reaction mixture was stirred for 30 min at 0 °C and 60 min at room temperature. The solvent

was removed under reduced pressure and the obtained oily residue purified with flash chromatography (neutral Al_2O_3, Ethylacetate: Hexane = 3 : 1) to afford 1.26 g of (4*R*,5*R*,E)-allyl 3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-ethylidene-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (**4**); yield 82%, yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.06 (d, 3H, *J* = 7.2 Hz), 1.52–1.66 (m, 1H), 1.84 (d, 3H, *J* = 7.2 Hz), 2.79–2.86 (m, 1H), 2.83 (s, 3H), 3.02 (s, 3H), 3.08–3.18 (m, 1H), 3.58–3.72 (m, 1H), 3.74–3.88 (m, 1H), 4.42–4.52 (m, 2H), 4.70–4.81 (m, 2H), 4.92 (d, 1H, *J* = 9.3 Hz), 5.13–5.24 (m, 2H), 5.43 (dd, 2H, J_1 = 17.1 Hz, J_2 = 1.8 Hz), 5.88–5.98 (m, 2H), 6.47–6.53 (m, 1H), [peaks for H2'α and H6 are under signal for H_2O]. ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.1, 17.1, 35.1, 36.0, 39.9, 40.8, 44.3, 54.1, 55.7, 61.3, 65.8, 66.2, 117.8, 118.6, 129.6, 131.4, 132.6, 138.8, 146.1, 153.9, 160.6, 168.4, 170.8. LC-MS: m/z calc for $C_{24}H_{31}N_3O_6S$: 489; obtd. 490 (M+H). MS m/z (relative intensity): 490 (M+H, 20), 279 (100), 154 (36). HRMS–FAB (*m/z*): calcd for $C_{24}H_{32}N_3O_6S$ (M+H), 490.2012; found, 490.2031. HPLC: 92.3 area %.

Synthesis of (4*R*,5*R*,E)-3-[(3*S*,5*S*)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-ethylidene-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (5). A solution of (4*R*,5*R*,E)-allyl 3-[(3*S*,5*S*)-1-(allyloxycarbonyl)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-ethylidene-4-methyl-7-oxo-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylate (**4**) (0.083 g), $Pd(PPh_3)_2Cl_2$ (2.5 mg) in dry dichloromethane (5 mL) was cooled to 0 °C. Argon was bubbled into the reaction mixture followed by the addition of water (15 μL) and the addition of Bu_3SnH during stirring within 30 minutes in 5 portions (5 × 40 μL). The reaction mixture was stirred at room temperature for 15–30 min and after the complete conversion was detected by HPLC, the reaction mixture was dried with $MgSO_4$ and the solvent removed under reduced pressure (T bath < 40 °C). The obtained oily residue was washed with diethyl ether (3 × 20 mL). The product was purified by the preparative HPLC and lyophilized at –20 °C to afford 0.041 g of (4*R*,5*R*,E)-3-[(3*S*,5*S*)-5-(dimethylcarbamoyl)pyrrolidin-3-ylthio]-6-ethylidene-4-methyl-7-oxo-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid (**5**), yield 66%, yellow solid; decomp at 160 °C (before mp). IR (KBr) 3436, 2967, 2931, 1752, 1701, 1653, 1608, 1508, 1446, 1381, 1244, 1137 cm^{-1}. $[\alpha]^{20}_D$ +23.5 (H_2O, 1.04) ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.05 (d, 3H, *J* = 7.2 Hz), 1.40–1.48 (m, 1H), 1.80 (d, 3H, J = 7.1), 2.52–2.60 (m, 1H), 2.80–2.86 (m, 2H), 2.83 (s, 3H), 2.96 (s, 3H), 3.05–3.08 (m, 1H), m for H2'α is under signal for water, 3.46–3.54 (m, 1H), 3.62–3.68 (m, 1H), 3.92–3.99 (m, 1H), 4.82 (d, 1H, *J* = 9.3 Hz), 6.42–6.45 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.7, 17.0, 35.1, 36.1, 42.8, 55.5, 57.8, 60.6, 128.0, 131.6, 139.0, 141.1, 164.1, 168.0, 171.7. HRMS–FAB (*m/z*): calcd for $C_{17}H_{24}N_3O_4S$ (M+H), 366.1486; found, 366.1507. HPLC: 92.6 area %.

4. 2. Enzymatic Assay

All the enzyme and compound solutions were prepared in 50 mM phosphate buffer pH 7.0. Assayed enzymes were incubated in presence of various concentrations of the tested compounds for 30 minutes at 30 °C in a final volume of 500 μl. The final concentrations of enzymes were as follows: TEM-1 (5 mg/ml), SHV-1 (0.5 mg/ml) and AmpC (1 mg/ml). Than 10 μl of 5 mM nitrocefin was added to the solution and the absorbance at 482 nm was measured for 5 minutes. The initial rate was determined and IC_{50} values were calculated from two independent experiments. The IC50 was recorded as the inhibitor concentration that give a initial hydrolysis rate of nitrocefin equal to 50% of the nydrolysis rate of nitrocefin in absence of inhibitor.

4. 3. MIC Determination

All tested bacterial strains were purchased from the American Type Culture Collection (ATCC): *E. coli* ATCC 25922 (S1), *E. coli* ATCC 35218 (S2), *S. aureus* ATCC 25923 (S3), *S. aureus* ATCC 43300 (S4), *S. aureus* ATCC 29213 (S5), *S. pyogenes* ATCC 19615 (S6), *E. faecalis* ATCC 2921 (S7), *H. influenzae* ATCC 49247 (S8).

Bacterial susceptibility, expressed as MIC, was determined by the microbroth dilution technique as recommended by the approved standard reference recommendations of the CLSI.[10] LK-175 was diluted by serial twofold dilution ranging from 128 μg/ml to 0.031 μg/ml.

The inoculum was prepared by the direct colony suspension method as described by CLSI.[10] Single colonies from the bacteria grown on blood agar plates at 35 °C for 24 h were suspended in a liquid medium to reach a final inoculum of approximately 5 × 10^5 CFU/ml. After the plates were incubated at 35 °C for 24 h, MICs were determined as the minimum inhibitory concentration [μg/ml] of antibiotic at which no visible bacterial growth occurred. Purity check and colony counts on each inoculum suspension was performed.

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6. References

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Povzetek

Razvoj novih inhibitorjev β-laktamaz z delovanjem proti klinično relevantnim sevom, ki proizvajajo β-laktamaze je eden najpomembnejših pristopov pri zagotavljanju klinične učinkovitosti β-laktamskih antibiotikov. 6-Hidroksietilno stransko verigo meropenema smo pretvorili v C6-etilidensko skupino z namenom, da ohranimo aktivnost proti β-laktamazam brez antibiotičnega delovanja. IC50 vrednosti C6-etiliden derivata proti TEM-1, SHV-1 in AmpC encimom so bile v spodnjem mM območju, kar je slabše kot pri meropenemu. Ob tej strukturni spremembi se je nepričakovano ohranila protimikrobna aktivnost, kar dokazuje, da C6-hidroksietilna stranska veriga ni nujni pogoj za ohranitev antibiotične aktivnosti meropenema.