Ampicillin permeation across OmpF, the major outer membrane channel in *E. coli.*

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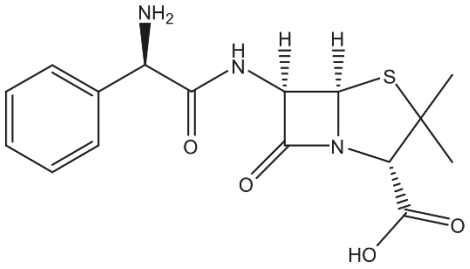
Supplementary information

Materials:

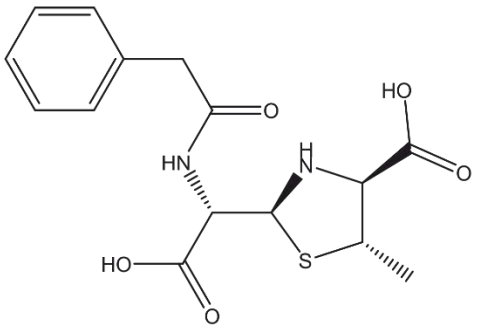
Materials: Ampicillin Sodium, Ampicillin (anhydrous basis), Sodium deuteroxide, Deutero-hydrochloric acid was obtained from Sigma Aldrich (Germany) and all other chemicals used were procured from AppliChem, Germany. 1,2-diphytanoyl-sn-glycero-3-phosphocholine was procured from Avanti Polar Lipids (Alabaster, AL).

Ampicillin



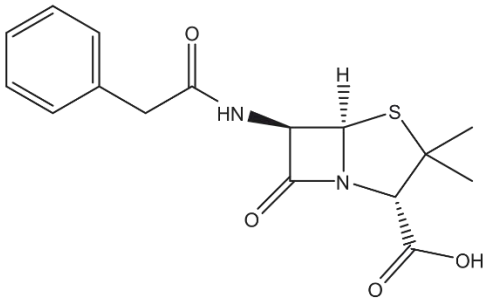
(2*S*,5*R*,6*R*)-6-((*R*)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

Penicilloic Acid



(2*S*,4*S*,5*S*)-2-((*S*)-carboxy(2-phenylacetamido)methyl)-5-methylthiazolidine-4-carboxylic acid

Benzylpenicillin



(2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

Figure S1. (Left) Ampicillin, (Right) Penicilloic Acid (1). (Bottom) Benzylpenicillin

Methods:

Planar Lipid Bilayer measurements:

Degradation of Ampicillin**:** Ampicillin was subjected to decomposition by incubating in aqueous solution at a concentration of 40 mM at pH ≈ 12.5 obtained by addition of 3M KOH after 45 min and adjusting back the pH to 6 using HCl. The overall pH for the ion-current measurements was strictly maintained at pH 6. For NMR measurements, the ampicillin was subjected to decomposition at concentration of 40 mM, pD about 12.5 for about 45 minutes using NaOD and

back titration to acidic pD using DCl. The concentration of the decomposed products for the electrophysiological measurements was adjusted equivalent to that of ampicillin to finally 20 mM (for more details see results section).

After incorporation of a single trimeric OmpF into the bilayer single channel currents were recorded at the given membrane voltage (V_m) (control) containing 1M KCl, buffered with 20 mM MES, followed by addition of 20 mM ampicillin added to the cis or trans compartment and the respective electrical ion channel currents were recorded. Subsequently, ampicillin was completely removed by perfusion of the cis or trans side (10-20 times exchange of the chamber solution 1M KCl with 20 mM) thereby preserving the bilayer containing the single trimeric OmpF channel. In the following, we then added the decomposed/ degradation ampicillin product to the cis or trans side of the bilayer corresponding to the initial concentration of 20mM ampicillin before the alkaline induced decomposition (see above) and recorded the respective single channel currents. This procedure allowed measuring the effect of ampicillin and its alkaline induced decomposition products on the identical OmpF channel with the same relative orientation in the bilayer.

Reversal potential measurements

The current voltage relation of the individual experiments was calculated from single averaged currents increments at the given voltage. Standard solutions (blank) contained 30mM KCl, buffered with 10 mM HEPES, pH 8, cis/trans, (cis is ground connected side). Followed by blank current voltage relation curve, the substrate Ampicillin, Benzylpenicillin and Ampicillin degradation product penicilloic acid, was added to cis side (concentration provided in table 1). The relative permeability of cations vs anions vs substrate in the tri-ionic case (2,3) were obtained by fitting of the experimental I-V-curves with the Goldman-Hodgkin-Katz current equation [2](2,3). Special precautions have been taken to prevent the chemical decomposition of Ampicillin stock solutions at pH 8.

^{1}H-NMR spectra of ampicillin in D_20

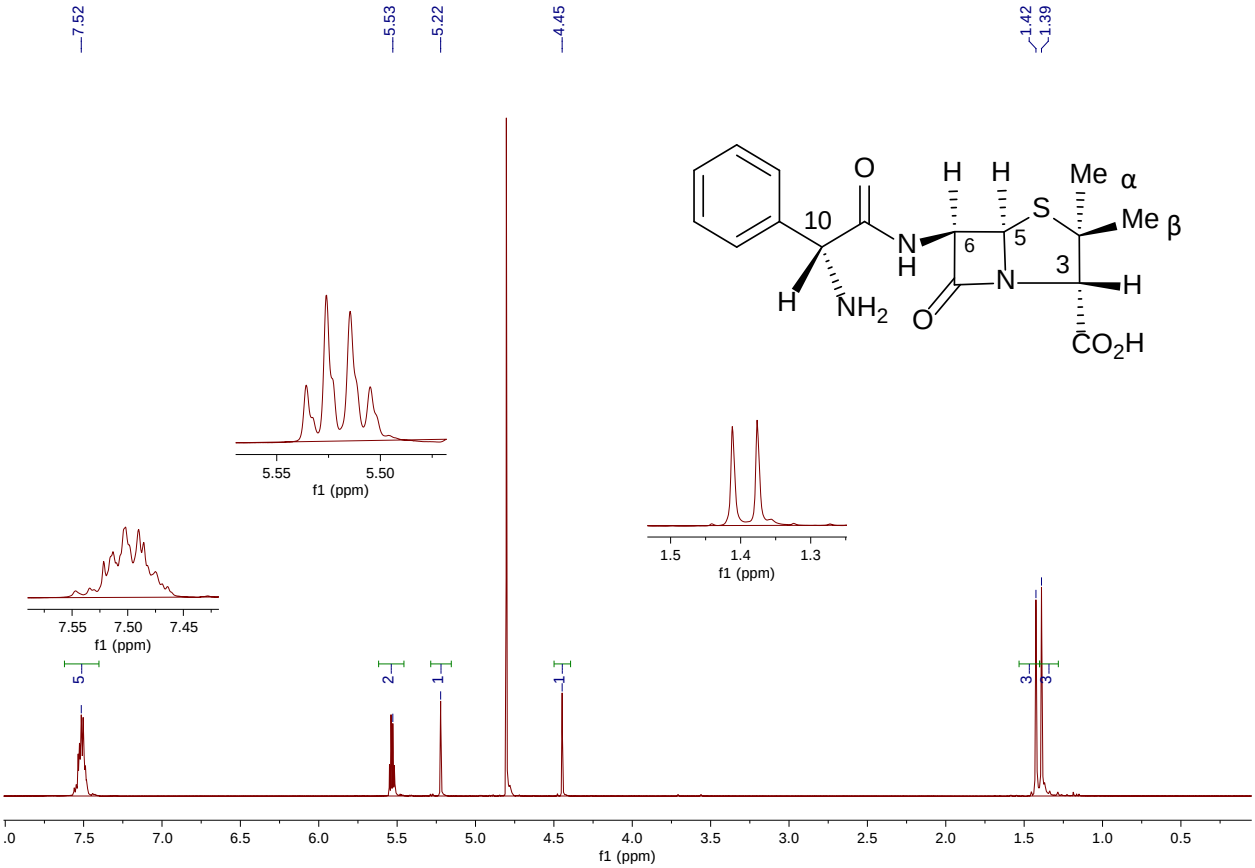


Figure S2: ^{1}H-NMR spectrum of 40mM ampicillin-Na in D_20 pD ≈ 4.

Measured ^{1}H-NMR spectra for 40mM ampicillin (Figure S2) in D_20 pD ≈ 4, ^{1}H-NMR (400 MHz, D_2O): $\delta = 1.39$ (s, 3H), 1.42 (s, 3H), 4.45 (s, 1H), 5.22 (d, 1H, $J = 4.87$ Hz), 5.53 (d, 2H, $J = 4.88$ Hz), 7.52 (m, 5H) ppm. Chemical shifts and assigned number of ^{1}H as obtained from the integration of the respective peak areas are indicated and summarized in (TableS1).

Compound	H-10 (ppm)	H-6 (ppm)	H-5 (ppm)	H-3 (ppm)	Me- β (ppm)	Me- α (ppm)	Phenyl group (ppm)
Ampicillin	5.2	5.5	5.5	4.4	1.4	1.3	7.5
Literature(4)	5.3	5.5	5.5	4.4	1.4	1.3	7.6

Table S1: Assigned ^{1}H-NMR chemical shifts of ampicillin-Na in D_2O in comparison with previously published values(4).

As obvious from (Table S1) the ^{1}H-chemical shifts and the relative peak areas for the individual ^{1}H of ampicillin-Na in D_20 compare well with the published values (4,5). Moreover, the ^{1}H-NMR -spectrum of ampicillin-Na in D_20 shows that the compound was pure, since no further not assignable ^{1}H-spectral lines were observed.

Alkali decomposition of Ampicillin

Ampicillin-Na in D_20 was adjusted to pD ≈ 12 by addition of NaOD for a period of approximately 45 to 60 min. followed by re-adjustment to of the pD, to $pD \approx 4$ by addition of DCl corresponding to a final ampicillin concentration of 40 mM. Subsequently the ^{1}H-NMR spectrum of the decomposed/ degradation ampicillin was recorded (Figure S3) and compared with ampicillin (Figure S4).

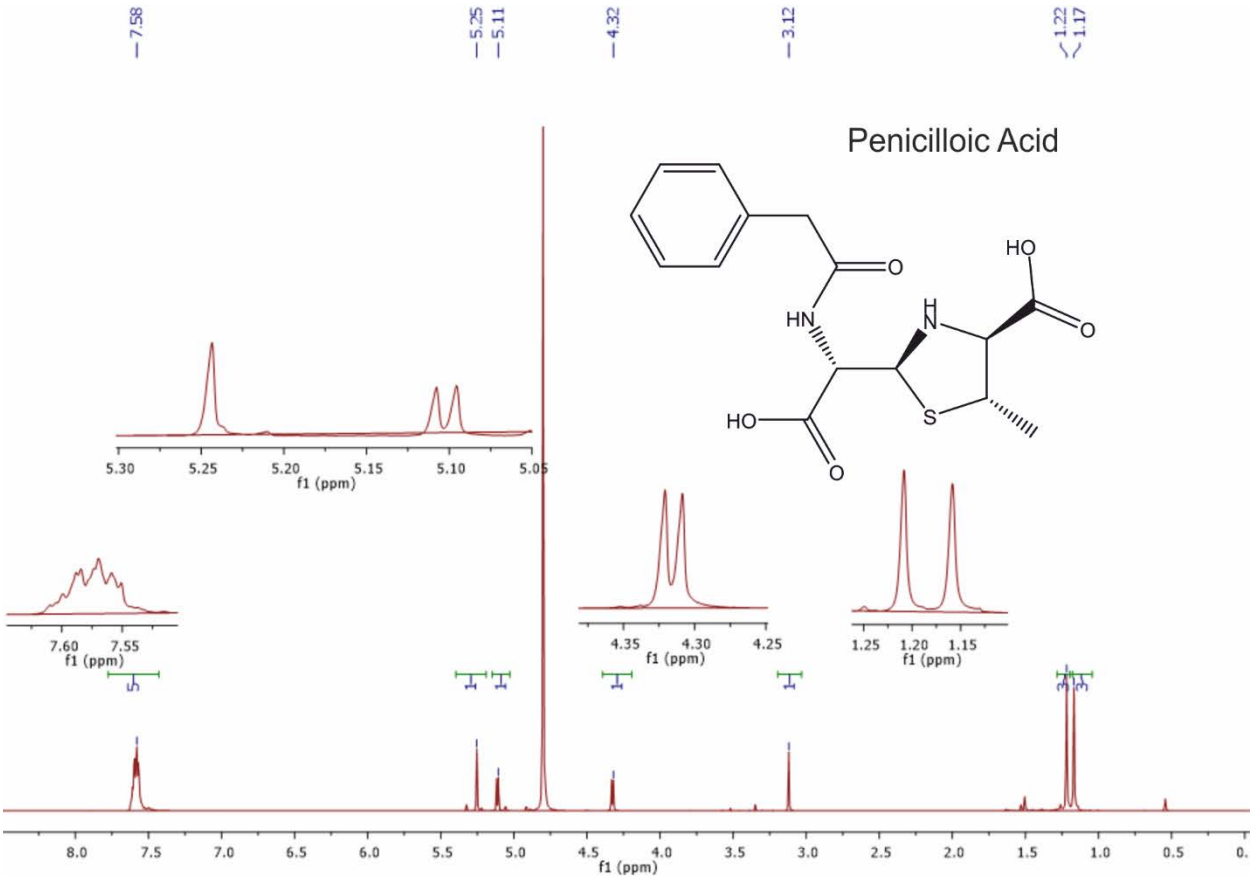


Figure S3: ^{1}H-NMR spectrum obtained after alkali decomposition/ degradation of 40 mM ampicillin-Na (pD=12 ± 1) measurement in D_20 pD ≈ 4.

Measured ^{1}H-NMR obtained after alkali decomposition/ degradation of 40mM ampicillin-Na at (pD ≈ 12) measurement in D20 pD ≈ 4, ^{1}H-NMR (400 MHz, D_2O): δ = 1.17 (s, 3H), 1.22 (s, 3H), 3.12 (s, 1H), 4.32 (d, 1H, *J* = 4.87 Hz), 5.11 (d, 1H, *J* = 4.88 Hz), 5.25 (s, 1H), 7.57 (m, 5H) ppm.

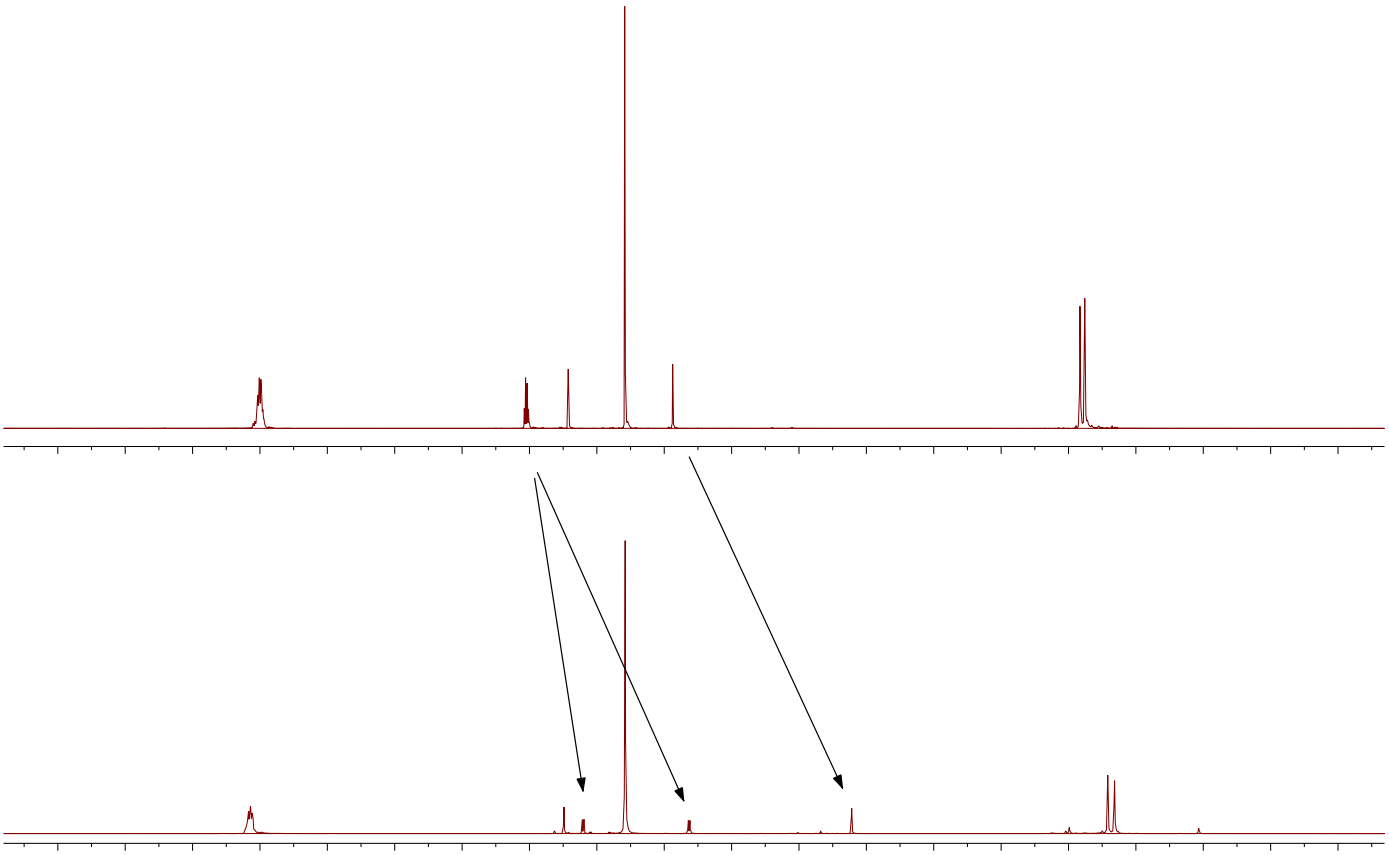


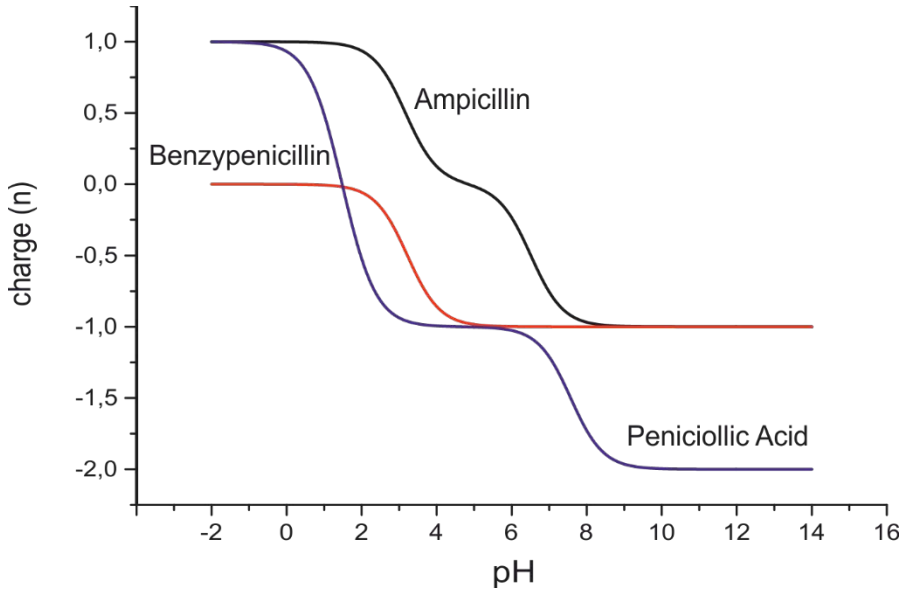
Figure S4: Comparison of the ^{1}HNMR spectra of ampicillin-Na and its alkali decomposition/ degradation product, (penicilloic acid), in D_20 pD ≈ 4.

Compound	H-10 (ppm)	H-6 (ppm)	H-5 (ppm)	H-3 (ppm)	Me- β (ppm)	Me- α (ppm)	Phenyl group (ppm)
Penicilloic-Acid (4,5)	5.1	5.0	4.2	3.0	1.1	1.1	7.5
Alkaline degradation product of Ampicillin	5.2	5.0	4.3	3.1	1.2	1.1	7.5

Table S2. ^{1}H chemical shifts in the ^{1}H NMR spectra of the alkali ampicillin decomposition/ degradation product in comparison with penicilloic(4) acid in D_20 pD ≈ 4.

The obtained ^{1}H chemical shifts in the ^{1}H-NMR spectrum of the ampicillin degradation product were significantly different from the once of ampicillin (see Figure S3, S4). These differences are evident by the splitting of the peak for ^{1}H5 and ^{1}H6 into two doublets, strong shift in the peak for the ^{1}H3 and the large chemical shift separation for Me-α/Me-β observed in the ^{1}H NMR spectra for the ampicillin decomposition product. These results show that after the alkaline incubation of ampicillin the ß-lactam ring was almost completely hydrolyzed. As shown previously (4) the observed ^{1}H- chemical shifts correspond well to the once of penicilloic-acid (4) which has been shown to be the first, main decomposition product of ampicillin upon alkali-induced decomposition (4),(6). According to the respective peak areas the yield of penicilloic acid after alkaline decomposition of ampicillin was ≥90% while the small remaining broad bands of the ampicillin ß-lactam-ring ^{1}H represented ≅7-8%. Additional formed non-charged low molecular weight hydrocarbon molecule could not been identified.

Charges of ampicillin, penicilloic-acid and benzylpenicillin at different pH values



FigureS5: pH dependent charges of ampicillin, penicilloic-acid, and benzylpenicillin.

Values for the pH depended charges of ampicillin, benzylpenicillin and penicilloic acid were calculated from the individual pKa values of the respective proteolytic groups (see Table S1) using the Henderson-Hasselbalch equation (7).

Table S1 Calculated pKa values for the proteolytic groups in ampicillin, penicilloic-acid, and benzylpenicillin

The pKa values of the proteolytic groups of the three different molecules were calculated using the Molecular Networks platform for predicting acid dissociation constants, aqueous solubility, and octanol/water distribution coefficients (pKa, logS and logP) based on the Molecular Networks chemo informatics platform MOSES. (www.molecular-networks.com/moses, Molecular Networks GmbH, Erlangen, Germany) (8). The calculated titration curve for ampicillin in Figure S5 resembles very close the one given in literature (9).

Table S1

	Ampicillin	**Penicicollic acid**	**Benzylpenicillin**
pK_1	Atom: 19, pKa:3.17211	Atom: 6, pKa=1.316	pKa = Atom: 12, pKa:3.22
pK_2	Atom: 26, pKa:6.51381		_____
pK_3	----------	Atom: 14, 14 pKa=1.646	_____
pK_4	----------	Atom: 8, pKa=7.5724	_____

Table S2

Charges (n) of at different pH obtained from Figure S5

compound	**pH4**	**pH6**	**pH7**	**pH8**
Ampicillin	**n=+0.13**	**n=-0.24**	**n=-0.75**	**n=-0.96**
Penicilloic Acid	**n=-0.99**	**n-1.03**	**n=-1.22**	**n=-1.80**
Benzylpenicillin	**n=+0.13**	**n=-0.99**	**n=-1**	**n=-1**

Dipole moments of ampicillin, penicilloic-acid, and benzylpenicillin

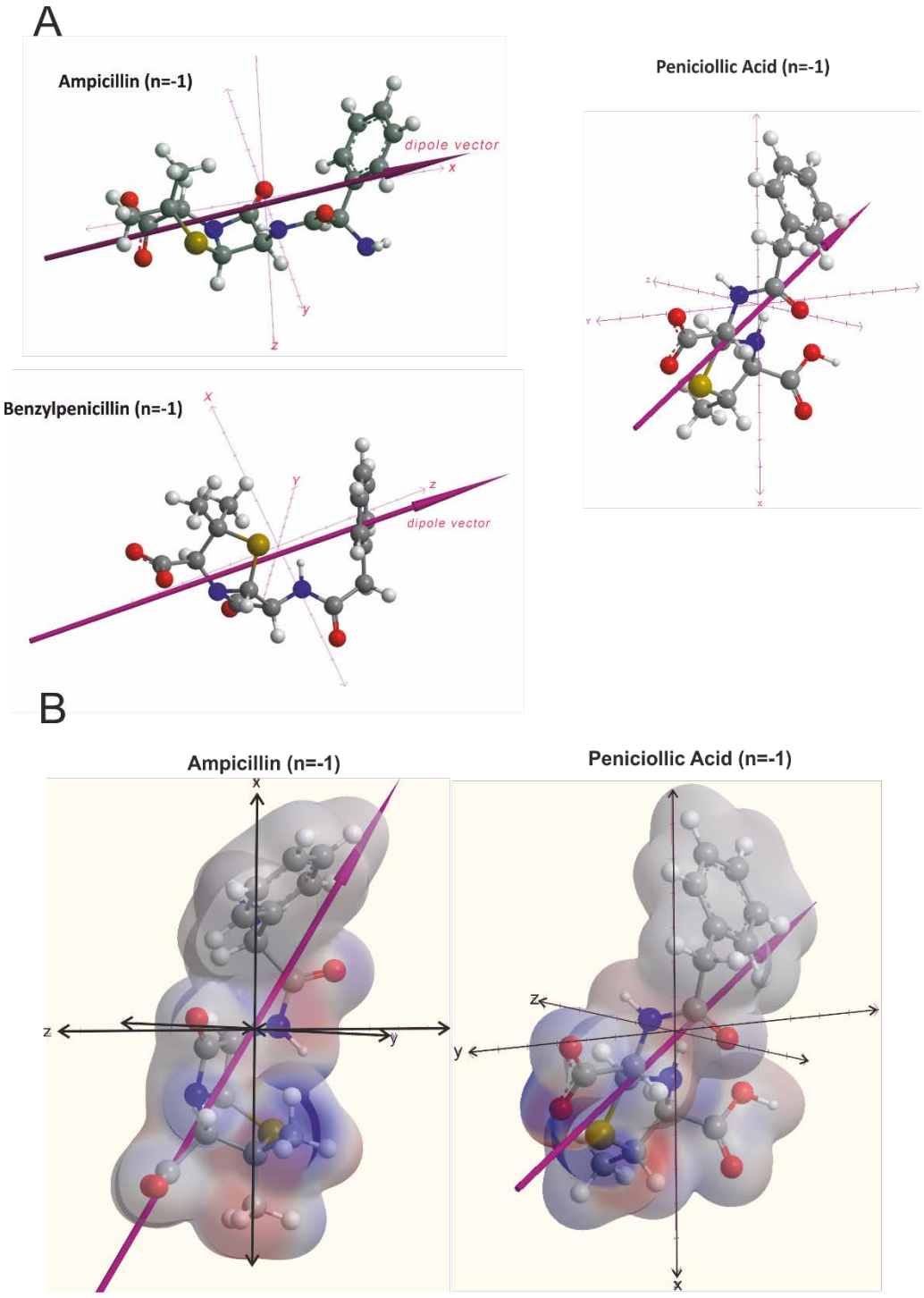
The electric dipole moment of the three different molecules were calculated using the MOPC program (10) in combination with the AM1 package (11).

Table S3

compound	X Dipole vector Debye	Y Dipole vector Debye	Z Dipole vector Debye	Total Dipole vector Debye	interface area (12) $Å^2$	Volume(12) $Å^3$
Ampicillin (n=-1)	15,109	5,987	-6,356	17,451	331.06	398,95
Penicilloic Acid (n=-1)	7.708	8.543	-7.659	13.822	330,79	381.27
Benzylpenicillin (n=-1)	2.549	3.081	3.016	5,089	347,10	389,76

After energy minimization of the individual molecules, using the AM1 package within the MOPAC program, ampicillin displays a somewhat elongated more prolate like shape with the orientation of the resulting dipole vector pointing rather parallel to the longer molecule axis (Figure S5). In contrast, after energy minimization, penicilloic acid and benzylpenicillin revealed a more donut like shape with the total dipole vector pointing almost orthogonal to the longer axis of the molecules (Figure S5). In summary; after geometrical energy minimization (MOPAC, AM1), the values for the interface surface and the volumes of the three different molecules are very close to each other, the main difference of the three molecules is net charge of the molecules at the given pH, the dipole strength, and the orientation of the dipole vector within the molecule coordinates (see Figure S6). Remarkably, considering the molecular surface obtained from extended Hueckel calculation and the overall shape of ampicillin (n=-1) and penicilloic acid (n=-1) appeared to be significantly different (Figure S6B). While ampicillin displays a prolate like volume structure with an 2D-projected axial ratio of $r \approx 1.7$, while penicilloic acid revealed a donate like volume shape with $r \approx 1.01$.

Figure S6: (A) Orientation of the dipole vectors of ampicillin, penicilloic-acid, and benzylpenicillin within the molecule coordinates. **(B)** Molecular surface obtained from extended Hueckel calculation and the overall shape of ampicillin (n=-1) and penicilloic acid (n=-1) with dipole vectors.



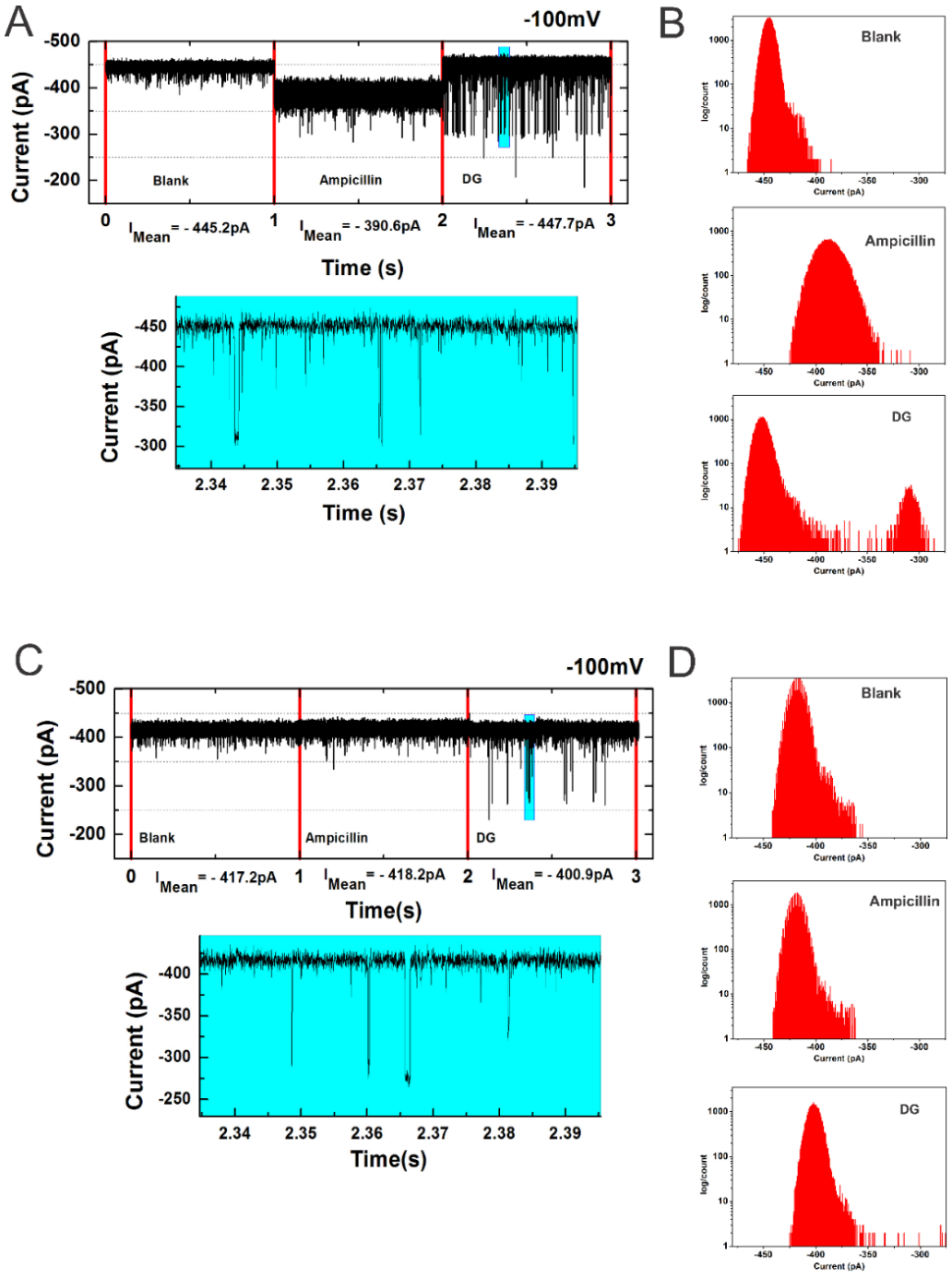


Figure S7: (A.) Ion channel current recordings from a bilayer containing a single reconstituted active trimeric OmpF, without addition (blank, lhs) and in the presence 20 mM ampicillin (Amp) and alkaline ampicillin degradation product; additions to cis side, applied potential -100 mV. (B.) Corresponding all point amplitude histograms. (C.) Ion channel current recordings from a bilayer

containing a single reconstituted active trimeric OmpF, no addition (blank. lhs) and in the presence 20 mM ampicillin (Amp) and alkaline ampicillin degradation product; additions to trans side, applied potential -100 mV. (D.) Corresponding all point amplitude histograms. Conditions: 1M KCl, buffered with 20mM MES, pH 6.0. OmpF was added to ground connected side, 20mM degraded ampicillin complex (*DG) is equivalent to 20 mM ampicillin.

Electrophysiological permeation assay: Description of the method

Determination of relative permeabilities

Our aim is to investigate to which extend the charged ampicillin/benzylpenicillin, which are available in limited quantities, are permeable through the OmpF channel. To resolve this issue, we apply as previously (2) an experimental electrophysiological tri-ionic zero-current-potential assay. Symmetric low salt concentrations on both sides of the OmpF containing membrane are supplemented with low concentrations of ampicillin with counter ion K^+ at pH 8 on one site of the membrane (tri-ionic conditions) and the reversal potentials are determined. This setup allows for resolving single channel currents in the range $\ll 100\,pA$. Using the determined zero-current-potential (V_{rev}) and applying the GHK-current equation (equation (1) below) (2,13) we then can determine the relative permeability of the involved ion-species (see below).

Experimental conditions for determining relative flux rates for ampicillin, K^+ and Cl^- at pH8:

Cation: $z_{K^+} = 1;\ c_{K^+cis} = 110\ mM; c_{K^+\,trans} = 30\ mM$

Anion: $z_{Cl^-} = -1.0;\ c_{Cl^-cis} = 30mM;\ c_{Cl^-trans} = 30mM$

Ampicillin: $z_{amp} = -0.96;\ c_{amp^-cis} = 80mM;\ c_{amp^-trans} = 00mM$

Zero current potential: $V_{rev} = 21.5\ mV\ (experimental\ value)$

Permeability: $P_{K^+} = 4;\ P_{Cl^-} = 1.0$ *(experimental value from bi-ionic recordings of KCl)*

Considering that the assumptions of the GHK-theory are valid at the given low ion concentrations and the ion fluxes can be regarded as independent of one another we can calculate the current voltage relation for a membrane channel with the above combination of bi-ionic and tri-ionic concentrations using equations 1 to 5 below:

(1) $$I_x(V, P_X, z, c_{cis}, c_{trans}) = P_x z^2 \frac{VF^2}{RT} \cdot \frac{\left(c_{x,cis} - c_{x,trans} exp\left(\frac{-zFV}{RT}\right)\right)}{1 - exp\left(\frac{-zFV}{RT}\right)}$$

(2) $$I_{K^+}(V) = I\left(V, P_{K^+}, z_{K^+}, c_{K^+cis}, c_{K^+trans}\right),$$

(3) $$I_{CL^-}(V) = I(V, P_{Cl^-}, z_{Cl^-}, c_{Cl^-cis}, c_{Cl^-trans})$$

(4) $$I_{amp^-}(V) = I\left(V, P_{amp^-}, z_{amp^-}, c_{amp^-cis}, c_{amp^-trans}\right)$$

(5) $$\sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) + I_{amp^-}(V)$$

The only unknown quantity in equations 1-5, the relative permeability of ampicillin (P_{amp^-}) is the variable, which can be used to fit the experimental I-V curve to finally obtain the experimental value of V_{rev} (see above). Because of the fit we obtained $P_{amp^-} = 0.75$. Thus, the resulting relative permeabilities at the applied tri-ionic conditions were: $P_{K^+} : P_{Cl^-} : P_{amp^-} = 4 : 1 : 0.75$

Determination of the ampicillin flux-rate through OmpF.

The determined relative permeability's can then be used subsequently to calculate the contribution of the individual ions at a given voltage to the total current (equation 1-4, see above). For this we calculate the I-V curves for the normalized total tri-ionic current ($\sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) + I_{amp^-}(V)$) and the bi − ionic current $\sum I(V) = I_{K^+}(V) + I_{Cl^-}(V)$. The difference of the two I − V relationships then yields the normalized $I_{amp^-}(V)$ (see Figure S8).

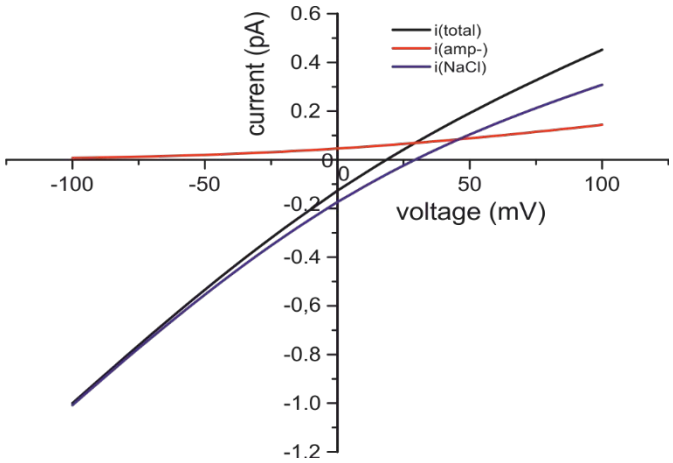


Figure S8: Calculated normalized current-voltage relation for a single OmpF-pore under the above given tri-ionic conditions.

Subsequently the known bi-ionic single pore conductance of OmpF can be used to “assign” the I-V relation to a channel pore with known conductance at the given ion concentrations (see Figure S8 and S9).

It is of interest to know the magnitude of the ampicillin current through a single OmpF pore at low µM concentrations and at low electrical driving force, i.e. at $V_m \leq 10mV$, since this value approaches in principle to the rate of the chemical gradient-driven transport. We can easily obtain this value from the resolved I-V curve by calibrating the separated normalized currents of the bi-ionic conductance of a single OmpF pore (G_{sp}) to the given KCl concentration from the difference of the tri-ionic minus bi-ionic I-V curve (Figure S8). We than can calculate the current-voltage relation for ampicillin and the OmpF conductance for ampicillin at the given concentrations. At 80 mM ampicillin (cis, pH 8) with K^+ as counter ion we obtain a value of $G_{amp}^{sp} \approx 8.3\ pS$. The turnover number (n) of ampicillin under this condition can then be calculated by: $n = \frac{G_{amp} \cdot V_m \cdot N_A}{F}$, ($N_A$= Avogadro-number and F= Faraday-constant). From this, we finally obtain at $V_m = +10mV$ a turnover number of of $n \approx 5.2 \cdot 10^5\ molecules/s$ for the ampicillin anion. Analogue calculations for a more physiological situation (symmetrically 100 mM KCl,cis/trans, pH 8 and 10 µM ampicillin cis) yielded a OmpF conductance of $G_{amp}^{sp} \approx 3.8\ fS$ (Figure S8 and S9). At $V_m = +10\ mV$ this results in turnover of ampicillin of $n \approx 237\ molecule/s$.

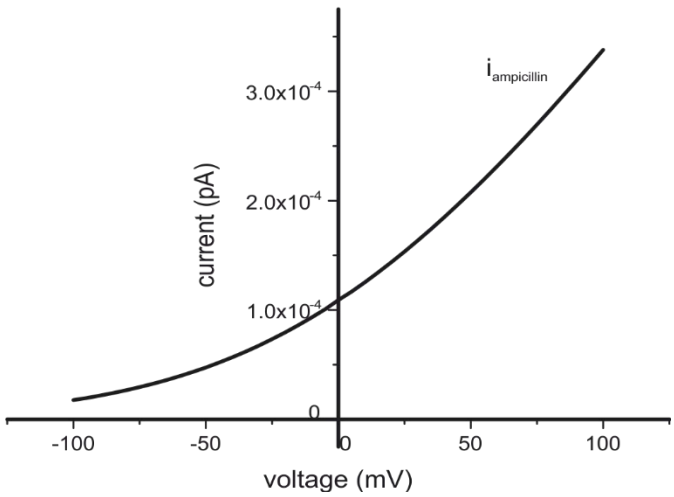


Figure S9:) Calculated current voltage relation for a single OmpF-pore bathed in 100 mM KCl symmetrical (cis/trans), pH 8 and 10 µM ampicillin (cis). Only the current carried by ampicillin anions is shown.

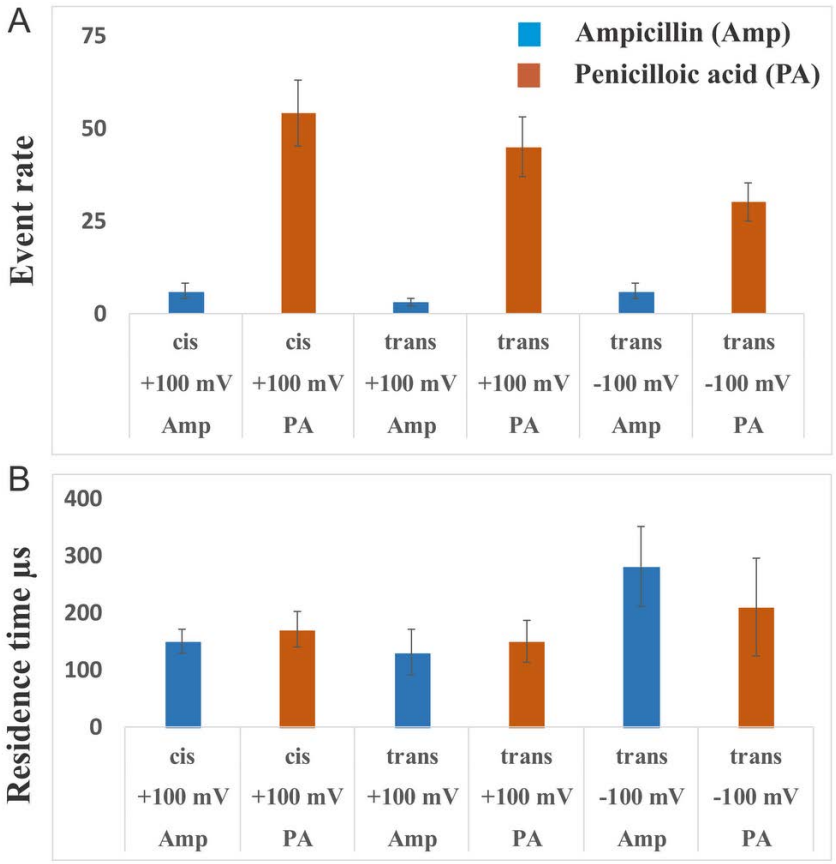


Figure S10: Analysis of single channel current blocking events **(A.)** Number of gating events/s (event rate) after addition of penicilloic acid or ampicillin to the indicated compartment at the indicated membrane potential $V_m = \pm 100\ mV$ **(B.)** Corresponding blocking residence time of OmpF in presence of ampicillin (Amp) and penicilloic-acid (PA) comparison. **Conditions:** 1M KCl, buffered with 20 mM MES, pH 6.0. OmpF was added to (cis = Ground connected side), 20mM degraded ampicillin complex (*DG) is equivalent to 20 mM Ampicillin. Number of events calculated per OmpF monomer per second.

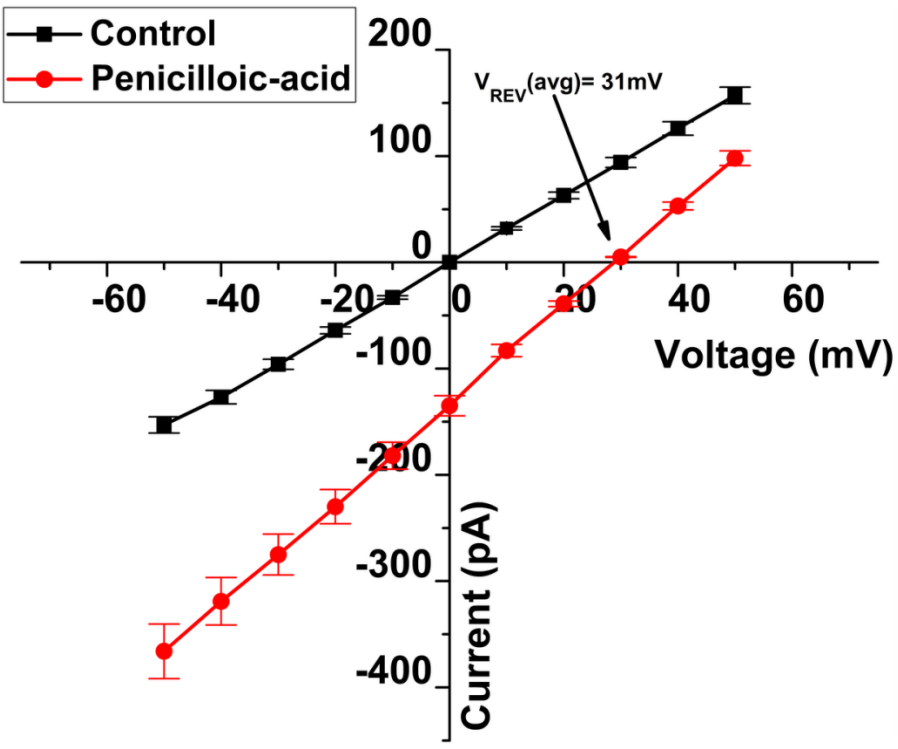


Figure S11: Current voltage–relation of reconstituted OmpF under symmetrical 30mM (cis/trans) bi-ionic conditions (control) and under tri-ionic conditions degradation product (penicilloic acid) (see Table 1). **Conditions:** 30 mM KCl, buffered with 10 mM MES, pH 6.0. OmpF was added to (cis = Ground connected side), ampicillin-degradation-product equivalent to ampicillin concentration was added to cis side.

Experimental conditions for determining relative flux for ampicillin degradation product penicilloic-acid:

Cation : $z_{K^+} = 1;\ c_{K^+cis} \approx 160\ mM; c_{K^+\ trans} = 30\ mM$

Anion: $z_{Cl^-} = -1.0;\ c_{Cl^-cis} \approx 100mM;\ c_{Cl^-trans} = 30mM$

Penicilloic-acid: $z_{degra} \approx -1.00;\ c_{degra^-cis} \approx 72mM;\ c_{degra^-trans} = 00mM$

Zero current potential: $V_{rev} = 31\ mV\ (experimental\ value)$

Permeability: $P_{K^+} = 4;\ P_{Cl^-} = 1.0$ *(experimental value from bi-ionic recordings)*

Because of the fit, we obtained $P_{penicill^{-1}} \leq 10^{-4}$ Thus, the overall relative permeabilities at the applied tri-ionic conditions were: $P_{K^+}: P_{Cl^-}: P_{degra^-} \approx 4: 1: < 10^{-4}$

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