Supplemental data

Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients

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MATERIALS AND METHODS

Reagents

The following products were purchased from Sigma-Aldrich (Gillingham, UK): Hanks balanced salt solution; penicillin-streptomycin; L-glutamine; HEPES; RPMI 1640; human AB serum; HSA (97-99%); and benzyl penicillin. Invitrogen (Paisley, UK) provided fetal bovine serum (FBS). Radiolabeled thymidine was obtained from Moravek International Limited (CA, USA). Trypsin was obtained from Promega (Madison, WI, USA).

Synthesis of penicillenic acid

The synthesis of PA was achieved by coupling oxazolone with D-penicillamine as described with modifications(Livermore et al., 1948). Briefly, the synthesis of PA **2** was achieved in four steps starting with a coupling of phenyl acetic acid **3** and glycine ethyl ester (Supplemental Figure 1A). The resulting ester **4** was then hydrolysed to acid **5** under basic conditions and then converted to the oxazolone **6**. The synthesis of oxazolone **6** was problematic due to the presence of a labile enol ether functional group. However, after much experimentation, we found that **6** can be prepared in reasonable yield and high purity by heating **5** with freshly distilled triethyl orthoformate in acetic anhydride, followed by a very rapid flash chromatographic separation. The following step, coupling of D-penicillamine with **6** in pyridine and triethylamine afforded the crude product **2**. A rapid recrystallisation with chloroform-hexane gave pure **2** as a pale yellow solid in 60% yield.

Ethyl 2-(2-phenylacetamido)acetate 6 2-phenylacetic acid (2.72g, 20mmol) was dissolved in dry DCM (30 ml) and treated with oxalyl chloride (5ml) and five drops of N,N-dimethyl formamide. The reaction mixture was stirred at room temperature for 2 hours, and then evaporated twice from dry DCM. The yellowish residue (acid chloride) was used directly in the next step.

To a solution of ethyl 2-aminoacetate (2.79g, 20mmol) in an ice cold, well stirred two phase mixture of 10% sodium bicarbonate solution (50 mL) and DCM (30 mL) was added the acid chloride solution (in 20 mL dry DCM) dropwise. Upon the completion of the addition, the reaction was allowed to stir for a further hour. Then the reaction mixture was diluted with ethyl acetate. The organic layer was washed with 1N HCl solution (20 mL), 10% sodium carbonate (20 mL) and brine, dried over anhydrous sodium sulphate and concentrated to afford **2** as a white solid (3.54 g) in 80% yield. Anal. ($C_{12}H_{15}NO_3$) Required C, 65.14; H, 6.83; N, 6.33%; Found C, 65.11; H, 6.86; N, 6.3%; ^{1}H NMR (400 MHz, $CDCl_3$) 1.26 (3 H, t, J = 7.1 Hz), 3.63 (2 H, s), 3.98 (2H, d, J = 5.2 Hz), 4.16 (2 H, q), 7.26-7.38 (5 H, m); ^{13}C NMR (100MHz, $CDCl_3$) 14.47, 41.87, 43.91, 61.86, 127.84, 129.42, 129.85, 134.89, 170.14, 171.41 *m/z* (CI, NH_3) 222 $[M+H]^+$, 100%).

2-(2-Phenylacetamido)acetic acid 7 Ethyl ester **5** was dissolved in ethanol and 1M sodium hydroxide solution (5 equiv.) and the reaction was stirred at 30ºC. When reaction was complete by TLC (3 hours), the solution was extracted with ethyl acetate (3x10 mL) to remove any organic impurities. The water phase was acidified using 1M HCl and extracted with ethyl acetate (3x20 mL). The combined organic phases were dried with anhydrous sodium sulphate and concentrated to give **6** as a white solid (g) in 80% yield. Anal. ($C_{10}H_{11}NO_3$)

Required C, 62.18; H, 5.74; N, 7.25%; Found C, 62.47; H, 5.89; N, 7.31%; ^{1}H NMR (400 MHz, MeOD) 3.59 (2 H, s), 3.92 (2H, s), 7.26-7.38 (5 H, m); ^{13}C NMR (100MHz, MeOD) 42.39, 43.96, 128.33, 129.97, 130.66, 137.05, 173.31, 174.86; *m/z* (CI, NH_3) 211 $[M+NH_4]^+$, 100%).

(E)-2-Benzyl-4-(ethoxymethylene)oxazol-5(4H)-one 6 Freshly distilled triethyl orthoformate (0.83 mL, 5mmol) was added to a mixture of acid **5** (0.486 g, 2.5 mmol) in 6 mL acetic anhydride. After stirring under N_2 at 120^{o}C for 1.5 hours, The reaction mixture was concentrated and separated by flash chromatography (10-15% ethyl acetate in hexane) to afford **6** as a pale yellow oil (0.27 g) in 47% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.6 (3H, t, J=7.2 Hz), 3.88 (2H, s), 4.32 (2H, q), 7.17 (1H, s), 7.24-7.34 (5H, m); ^{13}C NMR (100MHz, $CDCl_3$): δ_C 15.27, 35.9, 72.86, 116.77, 127.51, 128.82, 129.14, 133.38, 152.55, 162.34, 168.53; High-resolution MS (ESI) calculated for $C_{13}H_{13}NO_3$ $[M+H]^+$: 232.0974. Found: 232.0985.

(E)-2-{[2-Benzyl-5-oxooxazol-4(5H)-ylidene]methylamino}-3-mercapto-3-methylbutanoic acid 2 Triethylamine (1.6 mL) was added to a mixture of oxazolone **6** (0.226 g, 0.97 mmol) and D-penicillamine (0.145 g, 0.97 mmol) in pyridine (15 mL). The reaction was stirred under N_2 at 65^{o}C for 25 minutes and then concentrated. The residue was dissolved in chloroform (20 mL) and washed with pH 1.6 buffer (10 mL) and then pH 5.4 buffer (10 mL). The organic phase was dried over anhydrous magnesium sulphate and concentrated to afford the crude product as pale yellow oil. Recrystallization of crude product from chloroform and hexane yielded the PA **2** as a white solid (0.22 g) in 66% yield. ^{1}H NMR (400 MHz, $CDCl_3$) : δ_H 1.4 (3H, s), 1.6 (3H, s), 3.5 (2H, d, J=7Hz), 3.9 (1H,

s),7.24-7.34 (6H, m); ^{13}C NMR (100MHz, $CDCl_3$) : δ_C 28.9, 29.1, 35.1, 46.9, 73.2, 105.7, 127.6, 128.9, 129.2, 133.3, 142.6, 160.2, 167.1, 170.1;High-resolution mass (ES) calculated for $C_{16}H_{18}N_2O_4S$ $[M+H]^+$: 335.1066. Found: 335.1054.

Methods of mass spectrometric analyses

N-acetyl lysine adducts formed from BP or PA were separated on a Symmetry 5-µm C8 column (3.9 mm × 150 mm; Waters Corp, Milford, MS, USA) using a gradient of acetonitrile (10-50% over 20min) in 0.1% (v/v) formic acid with a flow rate of 0.9 mL/min. The analysis was carried out on a Quattro II instrument as described previously (Meng et al., 2007).

BP degradation products were analysed by LC-MS. Samples were separated on a prodigy 5 µm C18 column (150X4.6 mm, phenomenex) using a gradient of ACN (20%-80% over 18 min) in 0.1% formic acid with a flow rate of 1 mL/min. The samples were delivered into an API2000 triple quadrupole mass spectrometer (Applied Biosystems) and analysed as described previously (Jenkins et al., 2009). Transitions for multiple monitoring MS were selected based on experimental data. One MRM transition for BP was used, combining the m/z of BP (334 amu) with that of the dominant fragment ion (160 amu, [M+H]+ of thiazolidine ring).

Tryptic digests were reconstituted in 2% ACN/0.1% formic acid (v/v), and aliquots of 2.4-5 pmole were delivered into a QTRAP® 5500 hybrid quadrupole-

linear ion trap mass spectrometer (AB SCIEX) by automated in-line liquid chromatography (U3000 HPLC System, 5mm C18 nano-precolumn and 75 µm x 15cm C18 PepMap column [Dionex, California, USA]) via a 10µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 2% ACN/0.1% FA (v/v) to 50% ACN/0.1% FA (v/v) in 70mins was applied at a flow rate of 280 nL/min. The ionspray potential was set to 2,200-3,500V, the nebuliser gas to 18 and the interface heater to 150oC. Multiple reaction monitoring (MRM) transitions specific for drug modified peptides were selected as follows: the m/z values were calculated for all possible peptides with a missed cleavage at a lysine residue; to these were added the mass of the hapten (334 amu), the parent ion masses were then paired with a fragment mass of 160 ([M+H]+ of cleaved thiazolidine ring present in all of the haptens). MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity, they were optimised for collision energy and collision cell exit potential, and dwell time was 20ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit resolution, dynamic fill selected and dynamic exclusion for 20s. Total ion counts were determined from a second aliquot of each sample analysed by conventional LC-MS/MS and were used to normalize sample loading on column. MRM peak areas were determined by MultiQuant 1.2 software (ABSCIEX). Epitope profiles were constructed by comparing the relative intensity of MRM peaks for each of the modified lysine residues within a sample and normalization of those signals across samples.

Computer modellingof the non-covalent binding of BP and PA to albumin.

BP and PA were subjected to an *in silico* docking procedure using Autodock (Morris et al., 1998) and associated suite of programs. Autodock uses an empirical function to estimate the free energy of binding. This function contains five terms: a Lennard-Jones 12-6 dispersion/repulsion term; a directional 12-10 hydrogen bonding term; a screened Coulombic electrostatic potential; unfavourable entropy of binding due to restricted conformations and a desolvation energy term. For the calculations, the structure of HSA (PDB code 2BXM with myristate and indomethacin removed) was held rigid, and BP and PA were modelled in their neutral form. The centre of the volume that was searched for favourable binding poses was either Lys199 or Lys525 as appropriate. The grid spacing was 0.3 Å with 126 grid points in each direction. A distance dependant dielectric of -0.1465 was employed for all docking runs. A combination of a Lamarckian genetic algorithm and pseudo-Solis and Wets local search was used to generate docking poses for each molecule. The parameters used in this 'blind docking' procedure were those that have been shown to reproduce the binding mode of drugs within known structures of drug:crystal complexes with no prior knowledge of the binding site (Hetenyi and van der Spoel, 2002). The most popular docking pose for each molecule, as clustered by their RMSD, were identified and examined.

References:

Hetenyi C and van der Spoel D (2002) Efficient docking of peptides to proteins without prior knowledge of the binding site. Protein Sci 11:1729-1737.

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Meng XL, Maggs JL, Pryde DC, Planken S, Jenkins RE, Peakman TM, Beaumont K, Kohl C, Park BK and Stachulski AV (2007) Cyclization of the acyl glucuronide metabolite of a neutral endopeptidase inhibitor to an electrophilic glutarimide: Synthesis, reactivity, and mechanistic analysis. Journal of Medicinal Chemistry 50:6165-6176.

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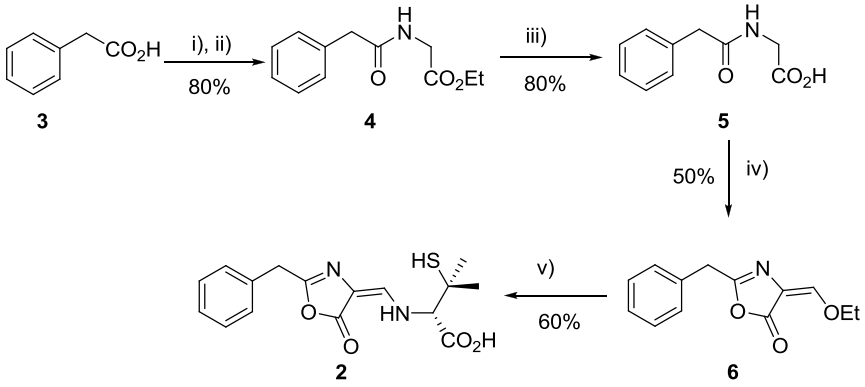
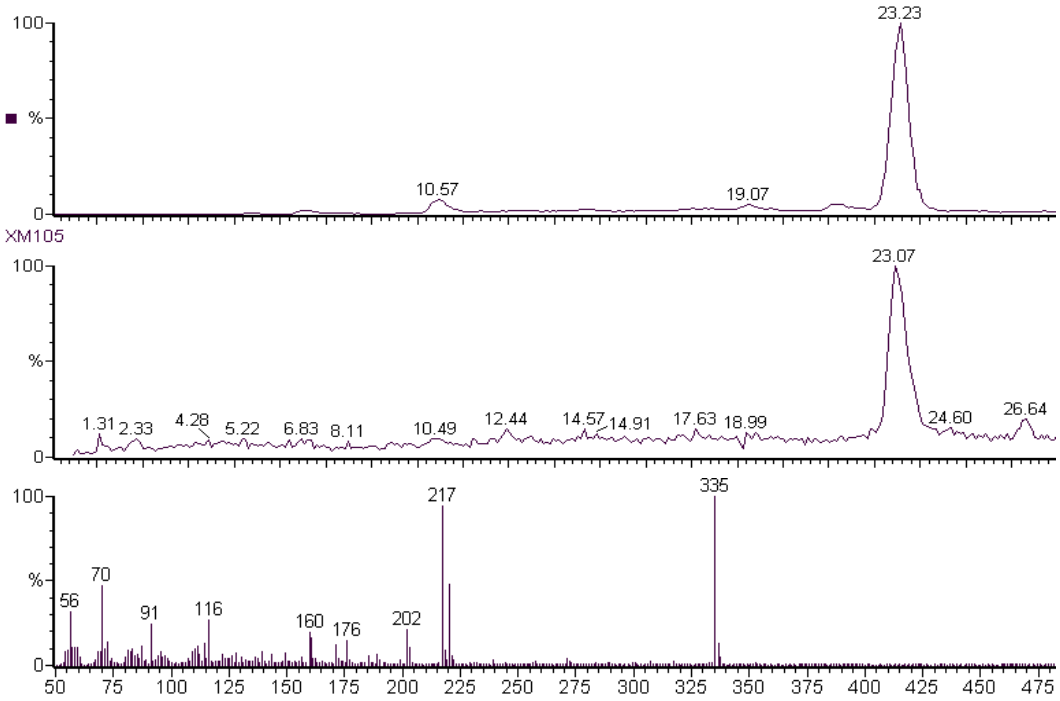


Reagents and conditions: i) $(COCl)_2$, DCM/DMF; ii) HCl $NH_2CH_2CO_2Et$; iii) 1M NaOH/ethanol, 35°C; iv) $HC(OEt)_3$, Ac2O, 120°C; v) D-penicillamine, Et_3N/pyridine

Supplemental Figure 1A. The synthesis of penicillenic acid



Supplemental Figure 1B. Characterisation of penicillenic acid

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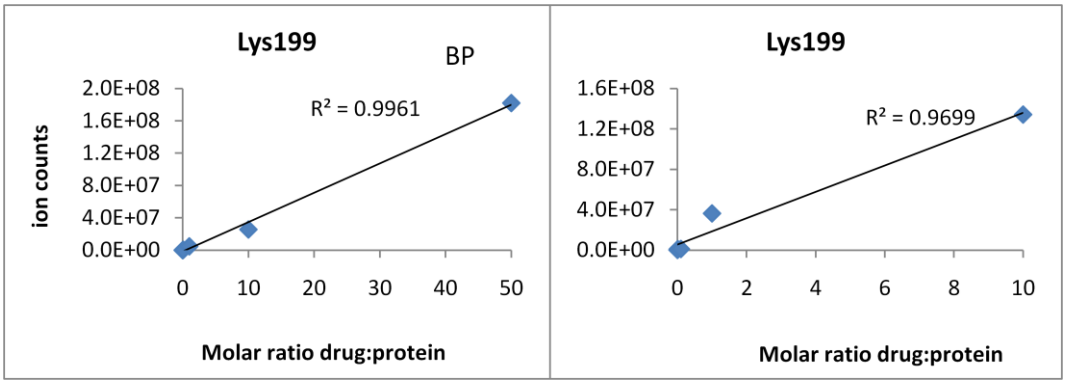
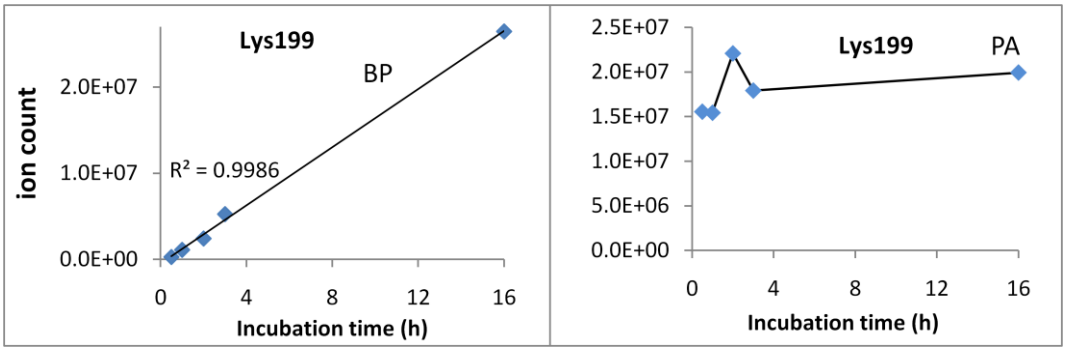
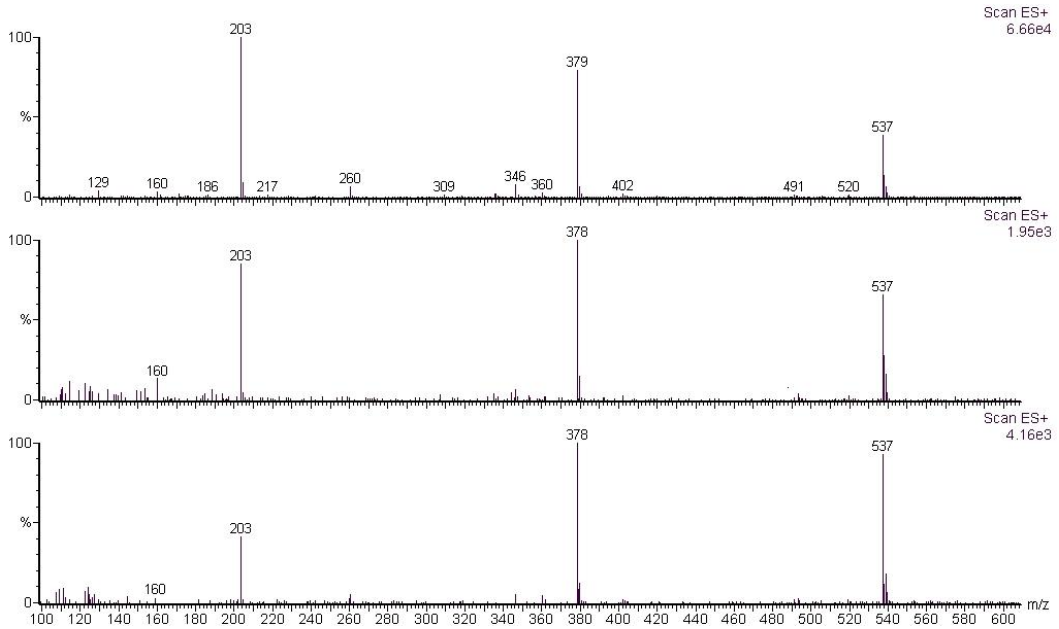
A



B



Supplemental Figure 2. Linear relationship between the level of modification and drug concentration (A) and incubation time (B).



Supplemental Figure 3. MS spectra of diastereomers of penicilloylated N-acetyl lysine methyl ester.

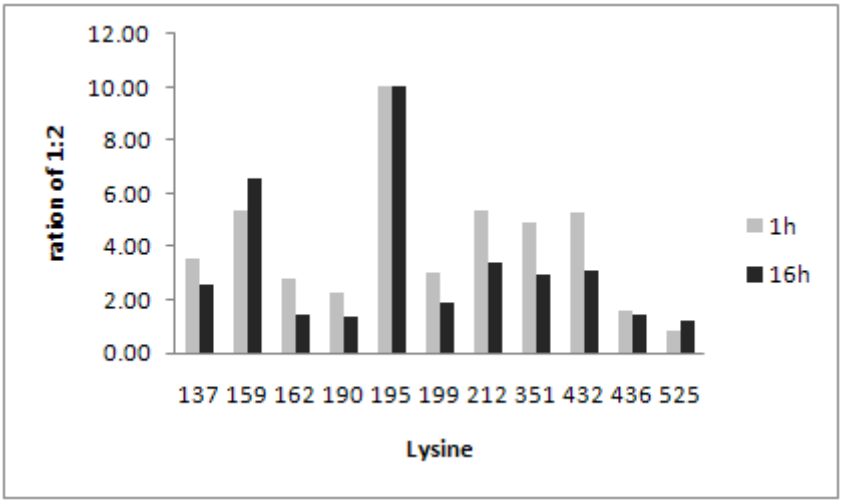
Supplemental Table 1 A. Diastereoisomeric penicillin hapten formed in the incubation of BP with HSA.

Peptide	Mass	1st peak RT	1st peak AUC	2nd peak RT	2nd peak AUC
KYLYEIAR	463.9	42.06	2.81E+05	44.09	1.14E+05
HPYFYAPELLFFAKR	745	50.6	1.90E+05	51.45	8.62E+04
YKAAFTECCQAADK	666.2	36.82	3.58E+05	38.24	1.45E+05
LDELRDEGKASSAK	618.3	31.83	1.94E+06	33.06	1.52E+06
ASSAKQR	541.2	26.7	5.58E+04	0	0
LKCASLQK	427.8	33.27	7.70E+07	34.61	3.24E+07
AFKAWAVAR	451.9	42.5	4.06E+05	44.26	1.28E+05
ATKEQLK	576.2	33.01	6.18E+05	34.5	3.65E+05
LAKTYETTLEK	544.2	37.3	3.53E+05	38.8	1.29E+05
NLGKVGSK	568.7	33.57	2.71E+05	35.53	9.85E+04
VGSKCCK	586.7	26.77	3.58E+04	28.96	1.56E+04
KQTALVELVK	488.2	43.01	7.75E+05	44.04	6.37E+05
EQLKAVMDDFAAFVEK	725.6	54.6	6.71E+05	------	0

Supplemental Table 1B. Diastereoisomeric penicillin hapten formed in the incubation of PA with HSA.

Peptide	Mass	1st peak RT	1st peak AUC	2nd peak RT	2nd peak AUC
AFKAWAVAR	451.9	42.5	4.02E+07	44.26	9.44E+07
ASSAKQR	541.2	26.7	2.93E+05	28.1	4.13E+06
ATKEQLK	576.2	32.93	1.62E+07	34.5	3.42E+07
EQLKAVMDDFAAFVEK	725.6	54.45	5.89E+05	-----	0
FKDLGEENFK	520.9	41.26	3.84E+06	42.38	1.01E+07
KQTALVELVK	488.2	43.01	7.98E+07	44.04	5.66E+08
KYLYEIAR	463.9	43.09	1.56E+07	43.96	2.46E+07
LAKTYETTLEK	544.2	37.3	7.65E+07	38.8	2.97E+08
LDELRDEGKASSAK	618.3	31.22	1.12E+07	32.73	6.44E+07
LKCASLQK	427.8	33.27	2.35E+07	34.61	5.14E+07
NLGKVGSK	568.7	33.15	9.07E+06	35.4	4.34E+07
VGSKCCK	586.7	26.25	0.00E+00	28.96	0.00E+00
VTKCCTESLVNR	600.9	35.73	5.98E+05	36.89	1.73E+06
YKAAFTECCQAADK	666.2	36.75	4.80E+05	38.1	1.32E+06
HPYFYAPELLFFAKR	586.7	50.6	5.43E+06	51.4	1.58E+07
VFDEFK*PLVEEPQNLIK	666.2	50.24	9.06E+05	51.51	1.78E+06

A



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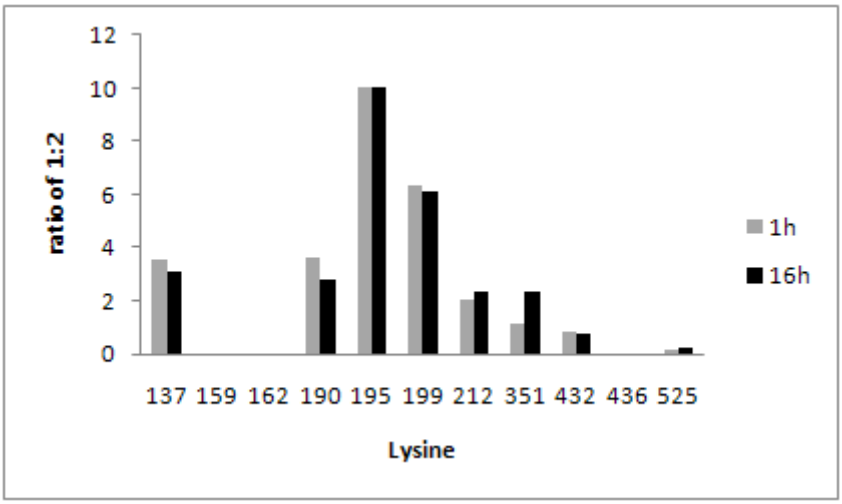

Supplemental Figure 4. The kinetic profile of distereoisomeric penicillin hapten formation in the continuous incubation of BP with HSA (A) and in the 'pulsed' incubation of BP with HSA (B).