学位論文

Development of potent antipseudomonal β-lactams by means of polycarboxylation of aminopenicillins
（アミノペニシリンのポリカルボン酸化による抗緑膿菌性βラクタム剤の開発）

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Development of potent antipseudomonal β-lactams by means of polycarboxylation of aminopenicillins

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CONFLICT OF INTERET

The authors declare that there is no conflict of interests.

Abbreviations: Ac-Amox, acetylated Amox; Amox, amoxicillin ; DTPA, diethylenetriaminepentaacetic acid; DTPA-Amox, DTPA-modified amoxicillin; DTPA-$[Amox]_2$, DTPA-modified amoxicillin dimer; HPLC, high-performance liquid chromatography; LB medium, Luria-Bertani medium; LC, liquid chromatography; MA-Amox, maleated Amox; MIC, minimal inhibitory concentration; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MTT, 3-[4,5-dimethylthiazol-2-yl]-

66 2,5-diphenyltetrazolium bromide; PBPs, penicillin-binding proteins; PBS, phosphate-
67 buffered saline; SA-Amox, succinylated Amox
68
69 Data Availability Statement
70 Waliul Islam was added as a new co-author.
71

Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that presents a serious risk to immunosuppressed individuals and other extremely vulnerable patients such as those in intensive care units. The emergence of multidrug-resistant *Pseudomonas* strains has increased the need for new antipseudomonal agents. In this study, we synthesized a series of amino group-modified aminopenicillin derivatives that have different numbers of carboxyl groups and structurally resemble carboxypenicillin-ureidopenicillin hybrids, and we evaluated their antipseudomonal activities. Among the derivatives synthesized, diethylenetriaminepentaacetic acid (DTPA)-modified amoxicillin (DTPA-Amox) showed potent antipseudomonal activity not only against the laboratory strain PAO1 but also against clinically isolated *Pseudomonas* strains that were resistant to piperacillin and carbenicillin. DTPA-Amox had no obvious cytotoxic effects on cultured mammalian cells. In addition, in an *in vivo* model of leukopenia, DTPA-Amox treatment produced a moderate but statistically significant improvement in survival of mice with *P. aeruginosa* strain PAO1 infection. These data suggest that polycarboxylation by DTPA conjugation is an effective approach to enhance antipseudomonal activity of aminopenicillins.

KEYWORDS

Antibiotics, drug resistance, *Pseudomonas aeruginosa*, aminopenicillin, amoxicillin

1 INTRODUCTION

The rod-shaped Gram-negative bacterium *Pseudomonas aeruginosa* is a common cause of nosocomial infections [1]. Although this bacterial agent does not usually affect healthy people, it can multiply in any area of the body that has enough humidity to allow colonization [2]. For example, this pathogen is the dominant cause of life-threatening chronic lung infections in patients with cystic fibrosis [3, 4]. As an disturbing statistic, multidrug-resistant strains were found in about 13% of these cases, and pan-drug-resistant specimens that cannot be treated with any available antipseudomonal antibiotics have been increasingly reported in clinical settings [5, 6]. Antimicrobial resistance has resulted in seriously limited treatment possibilities for *P. aeruginosa* infections, thereby leading to a critical situation because of the 51,000 deadly healthcare infections per year in the United States [7–9]. The consequence is an urgent need to develop novel agents with effective antipseudomonal activity [10].

Various semisynthetic penicillin antibiotics have been designed and their antibacterial activities against *P. aeruginosa* have been investigated. Carbenicillin (Fig 1) is the first semisynthetic β-lactam antibiotic that showed clinically useful activity against *P. aeruginosa* [11, 12]. The importance of the carboxyl group in the 6-acyl moiety for antipseudomonal activity was proved by establishing the potent antipseudomonal activity of ticarcillin, which is another carboxypenicillin [13]. However, to our best knowledge, the effects of the location and/or the numbers of carboxylic acid moieties attached to penicillin were not fully elucidated. Piperacillin (Fig 1) is another semisynthetic β-lactam antibiotic with effective antipseudomonal activity [14]. Piperacillin is a member of the ureidopenicillin family—mostly ampicillin derivatives in which the amino group side chain is modified to create various cyclic

ureas [15]. Ureidopenicillin-like derivatives with an amino group side chain modification of ampicillin derivatives have reportedly demonstrated antipseudomonal activity [16-18].

In this study, we synthesized a series of amino group-modified aminopenicillin derivatives that have different numbers of carboxyl groups and that structurally resemble carboxypenicillin-ureidopenicillin hybrids (Figs 1 and 2), and we investigated their antipseudomonal activities. Among the aminopenicillin derivatives that we synthesized, diethylenetriaminepentaacetic acid (DTPA)-modified amoxicillin (DTPA-Amox) had potent antipseudomonal activity that was comparable to the activity of piperacillin against *P. aeruginosa* strain PAO1. Also, DTPA-Amox showed antibacterial activity against certain clinical isolates of *P. aeruginosa* that were resistant to piperacillin and carbenicillin. Our data suggest that polycarboxylation by DTPA conjugation is an effective approach to provide aminopenicillins with antipseudomonal activity. Thorough understanding of the mechanisms involved in polycarboxylation-induced enhancement of such antipseudomonal activity is necessary for the development of highly effective antipseudomonal agents.

2 MATERIALS AND METHODS

2.1 Bacterial strains, culture media, and chemicals

P. aeruginosa strain PAO1 was obtained from the National Institute of Technology and Evaluation (Tokyo, Japan). Clinical isolates of *P. aeruginosa* strains NM-1 to NM-5 were a kind gift from Prof. Katsunori Yanagihara (Nagasaki University Hospital); strains MR-1 to MR-16 were from Dr. Koichi Tanimoto (Gunma University); and strains 808-1 and 808-2 were obtained from Kumamoto University Hospital.

142 *Escherichia coli* BW25113 was from the National Institute of Genetics (Shizuoka,
143 Japan). *Acinetobacter baumannii* (JCM 6841) was from RIKEN BioResource Research
144 Center (Ibaraki, Japan). *Staphylococcus aureus* was purchased from the American Type
145 Culture Collection (Manassas, VA). All other Gram-negative (*i.e.*, *Serratia marcescens,*
146 *Klebsiella pneumoniae, Salmonella typhimurium, Proteus mirabilis*) and Gram-positive
147 (*i.e.*, *Bacillus subtilis, Streptococcus mitis*) strains used in this study were from our
148 laboratory stock. All bacteria except *B. subtilis* were grown in Luria-Bertani (LB)
149 medium (1% NaCl [Nacalai Tesque, Kyoto, Japan], 1% peptone [Nihon Seiyaku,
150 Tokyo, Japan], and 0.5% yeast extract [Oriental Yeast Co., Ltd., Tokyo, Japan]) and on
151 LB agar plates (1.5% agar [Nissui Pharmaceutical Co., Ltd, Tokyo, Japan]). *B. subtilis*
152 organisms were grown in brain-heart infusion (Becton Dickinson and Company,
153 Franklin Lakes, NJ, USA). All strains were stored at –80°C in glycerol stock until
154 thawed for use. Ampicillin, amoxicillin, carbenicillin, and tazobactam were purchased
155 from FUJIFILM Wako Pure Chemical Corporation Ltd (Osaka, Japan); piperacillin was
156 from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan); and DTPA anhydride was
157 from Dojindo Laboratories (Kumamoto, Japan). For the carboxypeptidase assay, N^{α},N^{ε}-
158 diacetyl-Lys-D-Ala-D-Ala synthetic peptide (Sigma-Aldrich, St. Louis, MO, USA) was
159 used as a substrate, and formic acid (FUJIFILM Wako Pure Chemical Corporation Ltd)
160 was used to stop the reaction. For high-performance liquid chromatography (HPLC),
161 liquid chromatography-mass spectrometry (LC-MS), and liquid chromatography-
162 tandem mass spectrometry (LC-MS/MS) analyses, formic acid and acetonitrile (Kanto
163 Chemical Co., Inc., Tokyo, Japan) were used as the mobile phases.
164
165 **2.2 Syntheses of amoxicillin derivatives**

Amoxicillin derivatives were synthesized by reacting amoxicillin with acid anhydrides in aqueous medium: 20 mM amoxicillin was reacted with 20 mM acid anhydrides in 100 mM sodium bicarbonate buffer, pH 8.5 at 37°C for 60 min. The reaction mixtures were subjected to preparative HPLC for purification of the amoxicillin derivatives. Preparative HPLC was performed by using the Agilent 1260 Infinity series equipped with a photodiode array detector and an automated fraction collector (Agilent Technologies, Santa Clara, CA, USA). Samples (1 ml) were injected onto a YMC-Triart C18 Plus column (4.6 × 250 mm; YMC Co., Ltd., Kyoto, Japan) at 35°C. Mobile phases A (H_2O + 0.1% formic acid) and B (acetonitrile) were used with a linear gradient of 0.2% B to 40% B for 22 min, with the gradient maintained at 40% B for 1 min, after which the gradient was decreased to 0.2% B in 1 min, *i.e.*, at a flow rate of 0.8 ml/min. Amoxicillin derivatives were detected at 254 nm. Formation of amoxicillin derivatives was confirmed by means of MS as described below. Peaks corresponding to amoxicillin derivatives were collected by using a fraction collector, and the fractions were subjected to lyophilization.

2.3 LC-MS and LC-MS/MS

Amoxicillin derivatives and cleaved peptides derived from the DacC reaction were analyzed by means of LC-electrospray ionization-MS with the Agilent 6460 Triple Quadrupole LC/MS system (Agilent Technologies). LC conditions used were as follows: column, YMC-Triart C18 Plus column (2.1 × 50 mm) (YMC Co.) for amoxicillin derivatives, YMC-Triart C18 Plus column (2.1 × 150 mm) (YMC Co.) for cleaved peptides; column temperature, 45°C; injection volume, 1 µl; mobile phases: A, H_2O + 0.1% formic acid, and B, acetonitrile; gradient (B concentration), for amoxicillin

190 derivatives: 0 min – 1%, 10 min – 80%, 10.1 min – 1%, 15 min – 1%; for cleaved
191 peptides: 0 min – 0.2%, 2 min – 0.2%, 10 min – 50%, 10.1 min – 0.2%, 15 min – 0.2%;
192 flow rate, 0.2 ml/min. General conditions used for electrospray ionization-MS were as
193 follows: nebulizer gas, nitrogen delivered at 50 psi; nebulizer gas temperature, 250°C;
194 capillary voltage, 3500 V; collision gas, G1 grade nitrogen (Taiyo Nippon Sanso Corp.,
195 Tokyo, Japan). Multiple reaction monitoring was used to quantify the analyses. The
196 multiple reaction monitoring parameters used for cleaved peptides were as follows:
197 precursor ion, 302 *m/z*; product ion, 213 *m/z*; fragmentor voltage, 90 V; collision
198 energy, 9 eV; polarity, positive.
199
200 **2.4 Infrared (IR) spectrometry**
201 Before measurement, all samples were kept in desiccator for 24 h with silica gel to
202 absorb the moisture. About 2 mg of samples (DTPA, amoxicillin, or DTPA-Amox in
203 powder) were placed on the small eyelet of IR spectrometer chamber (JASCO FT/IR-
204 67100, Jasco Corp., Tokyo, Japan). Then, FT-IR spectroscopy was performed with
205 attenuated total reflectance (ATR) at room temperature. The spectra were monitored
206 from wave number range of 4000 to 500 cm^{-1}.
207
208 **2.5 Growth inhibition assay**
209 Bacterial strains were cultured overnight at 37°C in specified testing media. The
210 overnight cultures were then diluted 1000-fold into fresh media supplemented with 20
211 mM sodium phosphate buffer (pH 7.4) (FUJIFILM Wako Pure Chemical Corporation
212 Ltd) to maintain the stability of DTPA-Amox. Diluted bacterial suspensions were plated
213 in a 96-well flat bottom microplate (0.1 ml/well) and were treated with various

214 concentrations of β-lactam antibiotics in the presence or absence of 200 μM tazobactam.
215 After overnight incubation at 37°C, bacterial growth was determined by measuring the
216 optical density at 655 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The
217 minimal inhibitory concentration (MIC) was determined when the absorbance of the
218 wells were 0.035, that was identical to empty well.
219
220 **2.6 Preparation of recombinant DacC**
221 *E. coli* genomic DNA corresponding to *dacC* was obtained by using polymerase chain
222 reaction and the primers *Sph*I-*dacC*-F (GGGGCATGC
223 GCGGAACAAACCGTTGAAGC GCCGA) and *Hin*dIII-*dacC*-R (GGGAAGCTT
224 TCATCCGCCCTCTTCCACATTTT CCATCAC). The amplified DNA fragment was
225 cloned into the corresponding site of pQE80L (Qiagen, Hilden, Germany) (pQE-dacC),
226 and BL21(DE3)pLysS was transformed with pQE-dacC, which expressed *N*-terminal
227 His-tagged DacC. DacC was purified via the same protocol for purification of cysteine
228 synthase as reported previously [19]. The purified DacC concentration was measured by
229 means of the Bradford method (Nacalai Tesque), and the protein purity was determined
230 by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
231
232 **2.7 Carboxypeptidase assay**
233 Carboxypeptidase assays were carried out with the N^{α},N^{ε}-diacetyl-Lys-D-Ala-D-Ala
234 peptide (substrate peptide) (Sigma-Aldrich) as a substrate [20]. The substrate peptide
235 (final concentration, 100 μM) was reacted with 0.03 μg/ml purified His-tagged DacC in
236 50 mM Tris-HCl, pH 8.5, in the presence or absence of β-lactam compounds at 37°C for
237 30 min. Formic acid (final concentration, 0.1%) was added to the reaction mixtures to

terminate the carboxypeptidase reactions. N^{α},N^{ε}-Diacetyl-Lys-D-Ala peptide (cleaved peptide) was measured and quantified by means of LC-MS/MS analysis as described above.

2.8 Antimicrobial susceptibility test

The MIC of clinical isolates of *P. aeruginosa* was determined by a broth microdilution assay, according to Clinical and Laboratory Standard Institute (CLSI) reference methods [21]. Antimicrobial susceptibility test of each strain was performed by means of ready-made dry plates (DP-45) (Eiken Chemical Co., Tokyo, Japan). Plates contained 22 antimicrobial agents, including piperacillin (PIPC), tazobactam (TAZ)/PIPC, cefepime (CFPM), ceftazidime (CAZ), CAZ/Dipicolinic acid (DPA), cefozopran (CZOP), gentamicin (GM), minocycline (MINO), doripenem (DRPM), amikacin (AMK), levofloxacin (LVFX), aztreonam (AZT), imipenem (IPM), IPM/DPA, meropenem (MEPM), MEPM/DPA, colistin (CL), tobramycin (TOB), ciprofloxacin (CPFX), sulfamethoxazole/trimethoprim (ST), sulbactam (S)/cefoperazone (C), and fosfomycin (FOM). The bacterial cultures were adjusted with saline to a density equivalent to 1.0 McFarland standard and 25 μL of the suspensions were added into 12 mL of Mueller Hinton Broth (Becton Dickinson and Company). One hundred microliters of the inoculum were added to each well (approximately 5×10^4 CFU/well). Plates were incubated for 18 h at 37°C and examined by visual observation.

2.9 Cytotoxicity assay

Human cervical cancer HeLa cells and mouse colon carcinoma C26 cells were cultured in Dulbecco's Modified Eagle's Medium (FUJIFILM Wako Pure Chemical Corporation

262 Ltd) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals) and
263 1% penicillin-streptomycin (Nacalai Tesque) in a 5% CO_2 humidified incubator at
264 37°C. Cells were plated in 96-well plates at a density of 1.3×10^4 cells/well and were
265 allowed to grow overnight. Cells were then treated with DTPA-Amox (0–400 μM)
266 overnight at 37°C. Cell viability was determined by means of the 3-[4,5-
267 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on
268 the mitochondrial reduction of MTT to formazan, according to the manufacturer's
269 instructions (Dojindo Laboratories). Absorbance at 570 nm was measured by using a
270 microplate reader (Bio-Rad).
271

272 **2.10 Therapeutic effect of DTPA-Amox against *P. aeruginosa* infection in a**
273 **leukopenic mouse model**

274 Four-week-old male ddY mice each weighing 18–22 g were purchased from Japan SLC
275 Inc. (Shizuoka, Japan) and were housed at the Center for Animal Resources and
276 Development, Kumamoto University. All personnel involved with the animal study
277 received the educational training lectures from the Animal Care and Use Committee in
278 Kumamoto University. All procedures were approved by the Kumamoto University
279 Ethics Review Committee for Animal Experimentation and were performed to
280 minimize the number of animals used and their suffering. The specific criteria (i.e.
281 convulsions, coma or acute body-weight loss of 20%) were defined in the present
282 experimental protocol as humane endpoints to determine whether animals should be
283 euthanized. Leukopenia was induced by means of an intraperitoneal injection of 250
284 mg/kg cyclophosphamide (Sigma-Aldrich) at 4 days before the bacterial infection,
285 according to previous literature [21]. Food intake was stopped 1 day before infection

286 and was allowed to resume 1 h after infection. Leukopenic mice received an
287 intraperitoneal inoculum of *P. aeruginosa* strain PAO1: 0.1 ml (5×10^5 CFU). At 10
288 and 60 min after infection, mice received intraperitoneal injections of 0.1 ml (20 or 50
289 mg/kg) of DTPA-Amox. The survival rate of the mice was monitored for 72 hours after
290 the bacterial infection at every 2-h interval. However, 23 of 26 mice died before
291 induction of apparent symptoms as the criteria for euthanasia. Survival curves were
292 constructed by using the Kaplan-Meier method, and statistical significance was
293 analyzed via the log-rank (Mantel-Cox) test with GraphPad Prism 7.0 (GraphPad
294 Software, La Jolla, CA, USA).
295
296 **3 RESULTS**
297 **3.1 Syntheses of amino group-modified amoxicillin derivatives**
298 We synthesized a series of amoxicillin derivatives by reacting amoxicillin with acid
299 anhydrides in aqueous media. Fig 2 provides the chemical structures of the derivatives
300 obtained in this study. Purities and identities of the derivatives were confirmed by
301 means of HPLC and MS (S1–7 Fig). Because DTPA anhydride possesses two
302 anhydride moieties, reaction of amoxicillin with DTPA anhydride resulted in the
303 formation of DTPA-Amox and DTPA-$[Amox]_2$ (Fig 3A). DTPA-Amox and DTPA-
304 $[Amox]_2$ were purified from the crude reaction mixture by using reverse-phase HPLC,
305 which resulted in a single peak with a purity higher than 98% (Fig 3B-D; S7 Fig). The
306 yields of amoxicillin derivatives on the basis of amoxicillin were determined to be 94%,
307 91%, 88%, 65%, and 41%, for Ac-Amox, SA-Amox, MA-Amox, GA-Amox, TML-
308 Amox, and DTPA-Amox, respectively.

309 As shown in S8 Fig, the strong absorption peak that assigned to the > C=O
310 stretching of β-lactam ring at 1776 cm^{-1}, aromatic ring (C = C, 1616 cm^{-1}), and > C=O
311 stretching of -COOH group at 1582 cm^{-1}, all corresponded to amoxicillin were
312 identified [23]. The DTPA-Amox showed a strong and broad absorption spectrum at
313 1500-1700 cm^{-1} which indicate the clear evidence of amide bond between DTPA and
314 Amoxicillin. Moreover, the > C=O stretching of β-lactam ring was identified in DTPA-
315 Amox conjugate that suggest the stable β-lactam ring in the conjugate. These data
316 further support the conjugation of DTPA on amino group of amoxicillin to form amide
317 bond.
318 We found that pH greatly affected the stability of DTPA-Amox in aqueous media
319 (S9 Fig). DTPA-Amox decomposed spontaneously during incubation at 37°C when it
320 was dissolved in H_2O or 0.1% formic acid (S9 Fig). DTPA-Amox was stable, however,
321 during incubation in sodium phosphate buffer with a pH of 7.4. As an important
322 finding, we observed enhanced antibacterial activity of DTPA-Amox when the pH of
323 the LB medium was adjusted to 7.4 with sodium phosphate buffer compared with LB
324 medium without the pH adjustment (S9 Fig). Therefore, additional studies were
325 conducted with LB medium at pH 7.4, adjusted by using the sodium phosphate buffer.
326
327 **3.2 Antipseudomonal activities of amino group-modified amoxicillin derivatives**
328 Piperacillin and carbenicillin were used as representative semisynthetic
329 antipseudomonal β-lactams. Piperacillin showed potent antibacterial activity against the
330 *P. aeruginosa* strain PAO1 even in the absence of tazobactam (Fig 4). Carbenicillin,
331 however, completely suppressed PAO1 strain growth in the presence of tazobactam,
332 whereas carbenicillin alone incompletely inhibited bacterial growth.

Consistent with previous reports [24], native amoxicillin failed to inhibit the growth of PAO1 (Fig. 4) but in the presence of tazobactam, native amoxicillin dose-dependently inhibited PAO1 strain growth, with complete inhibition at 200 μM. An interesting finding is that all amoxicillin derivatives synthesized in this study showed stronger antipseudomonal activities compared with native amoxicillin both in the absence and presence of tazobactam. Among the derivatives, maleated Amox (MA-Amox) and DTPA-Amox demonstrated the most potent inhibition of the growth of PAO1 in the absence of tazobactam (Fig 4). Anti-pseudomonal activity of DTPA-Amox in the presence of tazobactam was determined on the basis of MIC, and found to be one-half and equivalent to those of existing anti-pseudomonal agents piperacillin and carbenicillin, respectively, against *P. aeruginosa* PAO1. DTPA alone demonstrated no antipseudomonal activity with or without tazobactam. In addition, a 1:1 mixture of free DTPA and native amoxicillin showed similar effects on PAO1 growth as that found for native amoxicillin. These data suggest that direct conjugation of DTPA to the amino group of amoxicillin is necessary for the enhancement of the antipseudomonal activity that was observed for DTPA-Amox. Similar to DTPA-Amox, DTPA-conjugated ampicillin showed superior antipseudomonal activity compared with parental ampicillin (S10 Fig). The antibacterial effects of DTPA-$[Amox]_2$ against *P. aeruginosa* (Fig. 4) and other Gram-negative rods such as *E. coli* and *S. typhimurium* (data not shown), however, were weaker than those of DTPA-Amox. Therefore, we used DTPA-Amox the next experiments.

We then studied the antibacterial activities of amoxicillin derivatives against clinical isolates of *P. aeruginosa*. As shown in Supplementary Table 1, almost all clinical isolates showed resistance against all types of antimicrobials including

357 carbapenems, cephems, aminoglycosides, fosfomycin, tetracycline, and others.
358 Carbapenem and cephem sensitivities were markedly recovered in the presence of
359 dipicolinic acid, a metallo-β-lactamase (MBL) inhibitor, indicating that clinical isolates
360 used in this study expressed MBLs. Fig 5 illustrates the effects of amoxicillin
361 derivatives synthesized in this study against clinical isolate MR13 as a representative
362 data. The growth of the clinical isolate MR13 was inhibited when 200 μM piperacillin
363 was added in the absence or presence of tazobactam. This result suggests that the
364 clinical isolate MR13 was more resistant than the PAO1 strain to piperacillin treatment.
365 MR13 was completely resistant to carbenicillin, succinylated Amox (SA-Amox),
366 acetylated Amox (Ac-Amox), and MA-Amox even in the presence of tazobactam. In
367 contrast, DTPA-Amox alone showed potent antibacterial activity against MR13. Table
368 1 summarizes the sensitivities of *P. aeruginosa* clinical isolates to piperacillin or
369 DTPA-Amox in the absence and presence of tazobactam. In the absence of tazobactam,
370 16 of 23 strains were resistant to both piperacillin and DTPA-Amox. Addition of
371 tazobactam potentiated the antipseudomonal activities of piperacillin and DTPA-Amox
372 against clinical isolates, although 9 isolates, as indicated by shaded cells in Table 1,
373 were resistant to piperacillin (minimum inhibitory concentration, >200 μM). DTPA-
374 Amox showed superior antibacterial activities compared with piperacillin against those
375 clinical isolates (Table 1). These data suggest that polycarboxylation that was achieved
376 by DTPA conjugation to aminopenicillins can enhance antipseudomonal activity,
377 especially for treatment of clinically isolated *P. aeruginosa* strains that were resistant to
378 piperacillin. It is also important to note that DTPA-Amox itself showed effective
379 bacterial killing against some clinical isolates such as NM4, NM5, and MR13. These

data may suggest that DTPA-Amox may be resistance against MBL-dependent degradation. Further study is needed to clarify this point.

3.3 Inhibition of penicillin-binding proteins by DTPA-Amox

The antibacterial mode of action of β-lactams relies on the ability of these agents to inhibit penicillin-binding proteins (PBPs). We studied the effects of DTPA conjugation to amoxicillin on the inhibition of PBPs. DacC (PBP6) cloned from *E. coli* (Ec-PBP6) was used as a model enzyme (S11 Fig). Enzyme activity was determined from the carboxypeptidase reaction that was monitored by detecting cleavage of the synthetic peptidyl substrate containing the D-Ala-D-Ala moiety (Fig 6A) [20]. The cleaved peptide formed from the reaction was detected and quantitated by means of MS/MS (Fig 6B). Addition of DTPA-Amox or native amoxicillin markedly inhibited the formation of the cleaved peptide released during the reaction (Fig 6C). A dose-response study suggested that DTPA-Amox can inhibit DacC to almost the same extent as native amoxicillin (Fig 6D). IC_{50} values were determined from the plotted figure to be 1.8 and 2.5 μM for amoxicillin and DTPA-Amox, respectively. DTPA alone, however, did not affect the reaction (Fig 6D). These data suggest that DTPA-Amox can inhibit the carboxypeptidase PBP activity, which may be attributed to the antipseudomonal activity of DTPA-Amox.

3.4 Antibacterial spectrum of DTPA-Amox

We then used Gram-negative and Gram-positive bacteria to investigate the effects of DTPA conjugation to amoxicillin on the antibacterial spectrum. As Fig. 7 illustrates, the effects of DTPA conjugation to amoxicillin differed depending on the bacteria. DTPA-

404 amoxicillin conjugation weakened the antibacterial activity against certain Gram-
405 negative rods including *E. coli*, *S. marcescens*, *P. mirabilis*, and *S. typhimurium*.
406 Antibacterial activities against *K. pneumoniae*, *A. baumannii*, and *S. aureus* were not
407 affected by DTPA conjugation. DTPA conjugation slightly enhanced the antibacterial
408 effects against *B. subtilis* and *S. mitis*.
409
410 **3.5 Effects of DTPA-Amox on mammalian cell viability**
411 We evaluated the cytotoxicity of DTPA-Amox to mammalian cells *in vitro*. Two
412 mammalian cell lines—human cervical cancer HeLa cells and mouse colon carcinoma
413 C26 cells—were treated overnight with DTPA-Amox, and cell viability was determined
414 by means of the MTT assay. As seen in Fig. 8, DTPA-Amox treatment did not have
415 significant cytotoxic effects on these cells at concentrations up to 400 μM. As noted
416 earlier, DTPA-Amox potently suppressed *P. aeruginosa* growth at concentrations less
417 than 200 μM (Figs 4 and 5 and Table 1), which indicates that DTPA can be used to treat
418 *P. aeruginosa* infections of mammalian cells.
419
420 **3.6 Therapeutic effects of DTPA-Amox on *P. aeruginosa* infection in a mouse**
421 **model of leukopenia**
422 Immunocompromised patients, including cancer patients, are highly susceptible to
423 bacterial infections caused by antibiotic-resistant Gram-negative bacteria such as *P.*
424 *aeruginosa* [25–28]. The DNA-alkylating agent cyclophosphamide-induced leukopenic
425 mouse has been used as an immunosuppressed host model for *in vivo* infection with *P.*
426 *aeruginosa* [22]. To develop the *in vivo* model for DTPA-Amox chemotherapy, we
427 treated ddY mice with an intraperitoneal injection of cyclophosphamide (250 mg/kg) 4

428 days before infection, as Fig 9A shows. We injected the PAO1 strain intraperitoneally
429 into this leukopenic model and monitored the survival rate. To determine whether
430 DTPA-Amox treatment was effective *in vivo*, mice were given DTPA-Amox (20 or 50
431 mg/kg) at 10 and 60 min after the bacterial challenge. In the present experimental
432 setting, all mice in the phosphate-buffered saline (PBS) control group died within 32
433 hours after the intraperitoneal PAO1 infection (Fig 9B). Although DTPA-Amox
434 treatment at 20 mg/kg tended to prolong the survival rate, no statistically significant
435 difference in survival rate was found compared with the control group ($p = 0.7141$). In
436 the group given the 50 mg/kg DTPA-Amox treatment, the survival rate was moderately
437 but significantly improved (Fig 9B, $p = 0.0395$ versus the PAO1 + PBS group). Three
438 of nine mice seemed to be cured by this treatment protocol at the end of experiment (72
439 hours), by which time they manifested fine coats and no apparent symptoms such as
440 shivering and convulsions. Our *in vivo* experiment indicated that DTPA-Amox
441 produced antipseudomonal activity *in vivo* and protected mice from the PAO1 infection.
442 This finding suggests a therapeutic potential of DTPA-Amox against *P. aeruginosa*
443 infection in immunocompromised patients.
444

445 **4 DISCUSSION**

446 Treatment of ampicillin and amoxicillin with acetic anhydride in slightly alkaline media
447 at room temperature has reportedly led to complete and instantaneous acetylation of
448 their amino groups [29–31]. This reaction is utilized to acetylate aminopenicillins
449 before imidazole- or 1,2,4-triazole-mercury(II) chloride-mediated derivatization of these
450 antibiotics for spectrophotometric determination [29–31]. In this study, we used a series
451 of acid anhydrides to modify the amino group of amoxicillin. Similar to acetic

452 anhydride, acid anhydrides used here readily reacted with amoxicillin in a
453 stoichiometric manner to form corresponding amino group-modified amoxicillin
454 derivatives (Fig 2). In the reaction of DTPA anhydride with amoxicillin, both DTPA-
455 Amox and DTPA-modified amoxicillin dimer (DTPA-[Amox]$_2$) were formed (Fig 3A).
456 Because DTPA-[Amox]$_2$ exhibited very weak antibacterial activity compared with
457 DTPA-Amox, we studied the antibacterial actions of DTPA-Amox in greater detail
458 here. DTPA monoanhydride is currently not commercially available; hence,
459 development of a synthetic method to generate DTPA monoanhydride may facilitate
460 large-scale production of DTPA-Amox.

461 To our knowledge, this report is the first to demonstrate that introduction of
462 carboxyl groups into aminopenicillins through amino group modifications via acid
463 anhydrides markedly improved antipseudomonal activity. Our data are consistent with
464 previous findings that amino group modifications of aminopenicillins enhanced
465 antipseudomonal activity [14, 16, 18, 32–35]. A noteworthy finding is that among the
466 derivatives synthesized in this study, DTPA-Amox had potent antipseudomonal activity
467 against clinical isolates of *P. aeruginosa* that were resistant to piperacillin and
468 carbenicillin (Fig 5, Table 1).

469 *P. aeruginosa* is an opportunistic human pathogen associated with an ever-
470 widening array of life-threatening acute and chronic infections, including ventilator-
471 associated pneumonia, urinary tract infections, bone and joint infections, bacteremia,
472 systemic infections, and infections associated with cystic fibrosis, otitis externa, and
473 burn and wound injuries [6, 36–38]. Nosocomial infections caused by *P. aeruginosa*
474 have become a healthcare concern, mainly because of the high level of resistance to
475 several antibiotics [39]. *P. aeruginosa* can develop resistance—a combination of

476 intrinsic, acquired, and/or adaptive resistance—to a wide range of antibiotics [6, 36–38].
477 The inducible expression of AmpC, a β-lactamase that degrades and inactivates β-
478 lactam antibiotics, is involved in the intrinsic resistance of *P. aeruginosa* to
479 aminopenicillins and cephalosporins, because these molecules induce the expression of
480 this β-lactamase [41]. Consistent with previous reports, the *P. aeruginosa* strain PAO1
481 used here was resistant to ampicillin and amoxicillin (Fig 4 and S9 Fig). Addition of the
482 β-lactamase inhibitor tazobactam at high concentration (200 μM; 60 μg/ml) moderately
483 enhanced the antipseudomonal actions of those aminopenicillins, which suggests that β-
484 lactamase (i.e., AmpC)-mediated inactivation may function, at least in part, in the
485 resistance of *P. aeruginosa* to those aminopenicillins under the current experimental
486 conditions. In contrast to aminopenicillins, piperacillin was found to possess low AmpC
487 inducer activity [40]. As a consistent result, the antipseudomonal activity of piperacillin
488 was apparently not affected by the addition of tazobactam. We also found that the
489 antipseudomonal activities of MA-Amox and DTPA-Amox were not affected by the
490 addition of tazobactam (Fig 4). This finding may suggest that MA and DTPA
491 modifications weaken the AmpC inducer activity of aminopenicillins, similar to actions
492 of piperacillin and other amino group-modified aminopenicillins such as apalcillin [42].
493 Carbenicillin was classified as having intermediate inducer activity [42]. We observed a
494 moderate enhancing effect of tazobactam on antipseudomonal activities of carbenicillin
495 and SA-Amox (Fig 4), which suggests that SA-Amox may behave as an intermediate
496 AmpC inducer.

497 Although both carbenicillin and piperacillin show potent antipseudomonal
498 activity, the antibacterial effects on other Gram-negative bacteria differ from each other.
499 For example, piperacillin had antibacterial activity against *Klebsiella* and *Serratia*

500 species, whereas carbenicillin was ineffective [14, 43]. In this study, we found that
501 DTPA conjugation markedly enhanced the antipseudomonal activity of
502 aminopenicillins. However, DTPA-Amox showed no antibacterial activity against *S.*
503 *marcescens* and *K. pneumonia* (Fig 7). Also as an important result, we found that
504 DTPA-Amox had antibacterial activity against certain clinical isolates of *P. aeruginosa*
505 that were resistant to piperacillin and carbenicillin (Fig 5, Table 1). These observations
506 suggest that DTPA conjugation can provide unique antipseudomonal activity not simply
507 because of amino group modifications or the introduction of a carboxyl group.

508 Patients who become neutropenic as the result of either underlying malignancy or
509 treatment with immunosuppressive agents are highly prone to *P. aeruginosa* infections
510 [25–28]. To study the therapeutic effects of DTPA-Amox on such disease conditions,
511 we used a cyclophosphamide-induced mouse model of leukopenia [22, 44]. Challenge
512 of these leukopenic mice by intraperitoneal *P. aeruginosa* injections have reportedly
513 caused sepsis because of an acute systemic infection, which led to the death of mice
514 within 1–3 days after infection [22, 45, 46]. Under the current experimental conditions,
515 all untreated mice died within 32 hours after infection (Fig 9). Statistically significant
516 protection was observed when mice were treated with DTPA-Amox at a dose of 50
517 mg/kg (Fig 9). Although this finding suggests the therapeutic potential of DTPA-Amox,
518 additional studies are needed to develop more successful treatments, and to determine
519 the effects of administration routes, dosages, and combinations with other type of
520 antibiotics.

521 In summary, we demonstrated that polycarboxylation of aminopenicillins can be
522 achieved by reacting the amino groups of the aminopenicillins with acid anhydrides. We
523 found that DTPA conjugation effectively produced potent antipseudomonal activity for

aminopenicillin-based β-lactams. It is noteworthy that DTPA-Amox was found to possess antibacterial effects against *P. aeruginosa* clinical isolates that were resistant against almost all types of antimicrobial agents. The different sensitivities observed for piperacillin and DTPA-Amox for clinical isolates of *P. aeruginosa* warrant continued investigation of the mechanisms involved in the enhancement of antipseudomonal activity by polycarboxylation.

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Figure Legends

Fig 1. Development of carboxypenicillin-ureidopenicillin hybrids by introduction of carboxyl groups into the amino acid side chains of ampicillin derivatives. R2, -H (ampicillin); -OH (amoxicillin).

Fig 2. Chemical structures of amoxicillin and its amino group-modified derivatives synthesized in this study.

Fig 3. Synthesis and characterization of DTPA-Amox. A, Synthetic pathway. B, Reverse-phase HPLC chromatogram for the reaction mixture of amoxicillin and DTPA anhydride. DTPA-Amox and DTPA-$[Amox]_2$ were eluted in Fraction 1 (Fr. 1; DTPA-Amox) and Fraction 2 (Fr. 2; DTPA-$[Amox]_2$) and were collected for lyophilization. C, Reverse-phase HPLC chromatogram of purified DTPA-Amox. D, Mass chromatogram of purified DTPA-Amox.

Fig 4. Antipseudomonal effects of piperacillin, carbenicillin, amoxicillin, and amino group-modified amoxicillin derivatives against *P. aeruginosa* PAO1. The *P.*

742 *aeruginosa* strain PAO1 was cultured overnight at 37°C in the presence of the indicated
743 concentrations of antibiotics in LB medium with the pH adjusted to 7.4. Bacterial
744 growth was determined by measuring turbidity at 655 nm absorbance. Data are means ±
745 SD ($n = 3$).

746 **Fig. 5 Antipseudomonal effects of piperacillin, carbenicillin, and amoxicillin**
747 **derivatives synthesized in this study against *P. aeruginosa* clinical isolate MR13**
748 **strain.** The MR13 *P. aeruginosa* clinical isolate was cultured overnight at 37°C in the
749 presence of the indicated concentrations of antibiotics in LB medium, pH 7.4. Bacterial
750 growth was determined by measuring turbidity at 655 nm absorbance. Data are means ±
751 SD ($n = 3$).

752 **Fig 6. Inhibitory effects of DTPA-Amox on the carboxypeptidase activity of DacC.**
753 A, DacC-mediated cleavage of the D-Ala-D-Ala-containing peptide. The cleaved peptide
754 can be quantitated by means of LC-MS/MS. Multiple reaction monitoring (MRM) of
755 cleaved peptides derived from the DacC reaction (B,C). D, Effects of amoxicillin,
756 DTPA-Amox, and DTPA on DacC-mediated cleavage of synthetic peptide. The DacC
757 reaction was carried out in the presence of the indicated concentrations of additives.
758 Data are means ± SD ($n = 3$).

759 **Fig 7. Antibacterial spectra of amoxicillin and DTPA-Amox**. Bacteria were cultured
760 overnight at 37°C in the presence of the indicated concentrations of amoxicillin or
761 DTPA-Amox, in pH-adjusted media. Bacterial growth was determined by measuring
762 turbidity at 655 nm absorbance. Data are means ± SD ($n = 3$).

763 **Fig. 8. Cytotoxicity of DTPA-Amox.** Viability of HeLa cells (A) and C26 cells (B)
764 treated overnight with the indicated concentrations of DTPA-Amox. The MTT assay
765 was utilized to determine viability. Data are means ± SD ($n = 3$).

766 **Fig. 9. Therapeutic effect of DTPA-Amox on *P. aeruginosa* infection in a mouse**
767 **model of leukopenia.** A, Time line of the *in vivo* treatment. B, Mice received
768 intraperitoneal injections of cyclophosphamide (Cy; 250 mg/kg) and were then infected
769 with *P. aeruginosa* strain PAO1 (5×10^5 CFU). At 10 and 60 min after infection, mice
770 received intraperitoneal injections of PBS (controls), 20 mg/kg DTPA-Amox, or 50
771 mg/kg DTPA-Amox. Survival of the mice was monitored for 72 hours after the

infection. PAO1 + PBS group $n = 8$; PAO1 + DTPA-Amox treatment groups $n = 9$. *$p < 0.05$.

Supplementary Figure Legends

S1 Fig. Characterization of Ac-Amox. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S2 Fig. Characterization of SA-Amox. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S3 Fig. Characterization of MA-Amox. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S4 Fig. Characterization of glutarated Amox (GA-Amox). A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S5 Fig. Characterization of trimellitated Amox (TML-Amox). A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S6 Fig. Characterization of DTPA-conjugated ampicillin **(DTPA-Amp).** A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S7 Fig. Characterization of DTPA-[Amox]$_2$. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.

S8 Fig. Infrared spectra of amoxicillin, DTPA-Amox, and DTPA.

S9 Fig. Effects of culture medium pH on the stability and antibacterial activities of amoxicillin and DTPA-Amox. A, Stability of DTPA-Amox in different media. DTPA-Amox was dissolved in H_2O, 0.1% formic acid, or 50 mM sodium phosphate buffer (pH 7.4), followed by incubation at 37°C for 2.5 or 4.5 hours. DTPA-Amox remaining in the medium was quantitated by means of HPLC. Antibacterial activities of native amoxicillin (B) and DTPA-Amox (C) against *E. coli*. LB medium was used without pH adjustment (pH 6.5) or with pH adjusted with sodium phosphate buffer (NaPB) to pH 7.4. Bacterial growth was determined by measuring turbidity at 655 nm absorbance after overnight culture. Data are means ± SD ($n = 3$).

S10 Fig. Antipseudomonal effects of (A) ampicillin and (B) DTPA-conjugated ampicillin**.** *P. aeruginosa* strain PAO1 was cultured overnight at 37°C in the presence of the indicated concentrations of antibiotics in LB medium at pH 7.4. Bacterial growth was determined by measuring turbidity at 655 nm absorbance. Data are means ± SD (n = 3).

S11 Fig. Purification of recombinant DacC by using nickel-nitrilotriacetic acid agarose affinity chromatography. A lysate of *E. coli* cells expressing DacC was subjected to nickel-nitrilotriacetic acid agarose affinity chromatography purification. Protein contents in each fraction were analyzed by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. Sup, supernatant; FT, flow through; E1-4, elution by imidazole-containing buffer at 50 mM (E1), 100 mM (E2), 200 mM (E3), or 300 mM (E4).

818 **Table 1. Minimum inhibitory concentrations (μM) of piperacillin and DTPA-Amox**
819 **for *P. aeruginosa* clinical isolates.**

Strain	Pip	DTPA-Amox	Pip/Tazo	DTPA-Amox/Tazo
PAO1	12.5	25	12.5	25
NM1	200	> 200	200	> 200
NM2	50	200	50	25
NM3	200	200	200	200
NM4	> 200	25	> 200	25
NM5	> 200	50	> 200	50
MR1	> 200	> 200	> 200	50
MR2	> 200	200	> 200	100
MR3	> 200	> 200	> 200	200
MR4	> 200	> 200	> 200	25
MR5	> 200	> 200	> 200	50
MR6	> 200	> 200	200	50
MR7	25	100	25	25
MR8	25	200	25	100
MR9	> 200	> 200	> 200	50
MR10	> 200	> 200	50	25
MR11	> 200	> 200	200	200
MR12	> 200	> 200	50	50
MR13	100	25	100	25
MR14	> 200	> 200	100	100
MR15	> 200	> 200	> 200	50
MR16	> 200	> 200	50	100
808#1	> 200	> 200	100	50
808#2	> 200	> 200	100	100

820 Pip, piperacillin; Tazo, tazobactam; DTPA-Amox, DTPA-conjugated amoxicillin.

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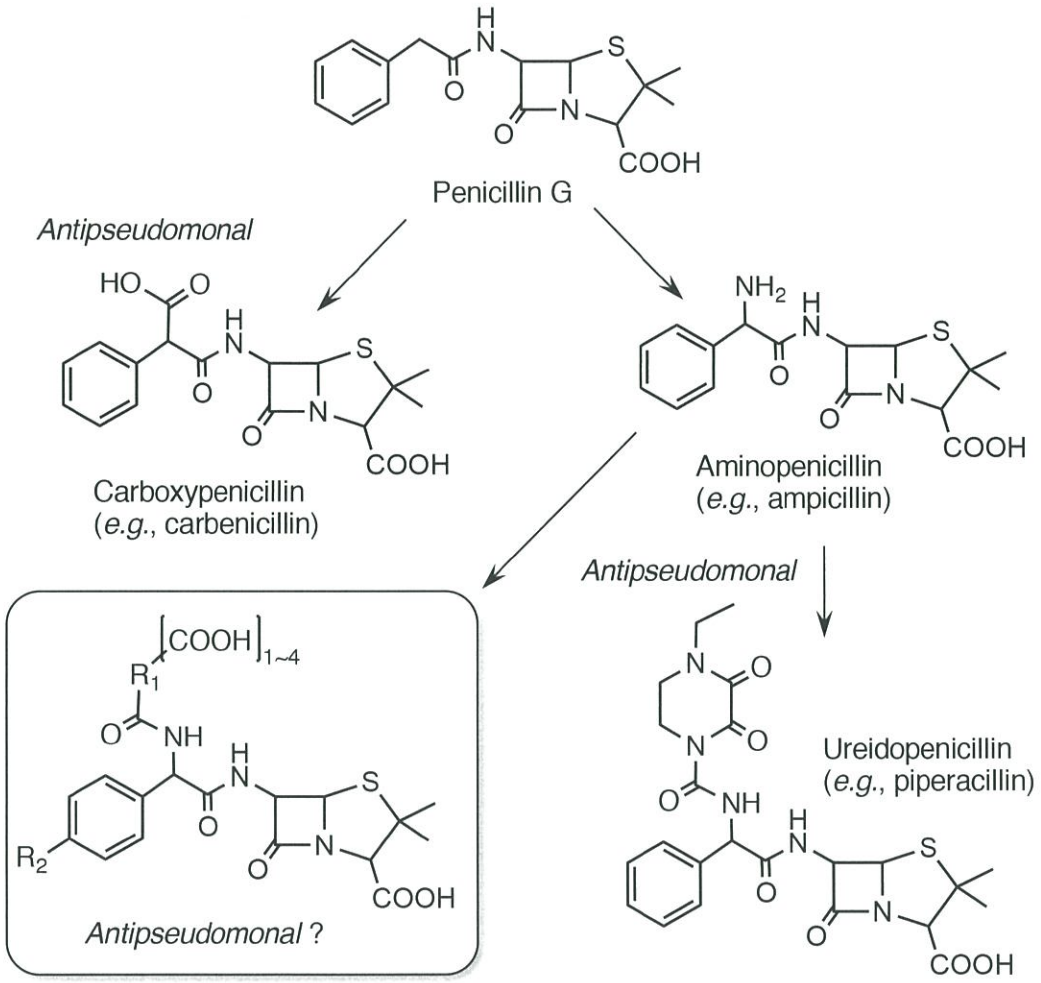
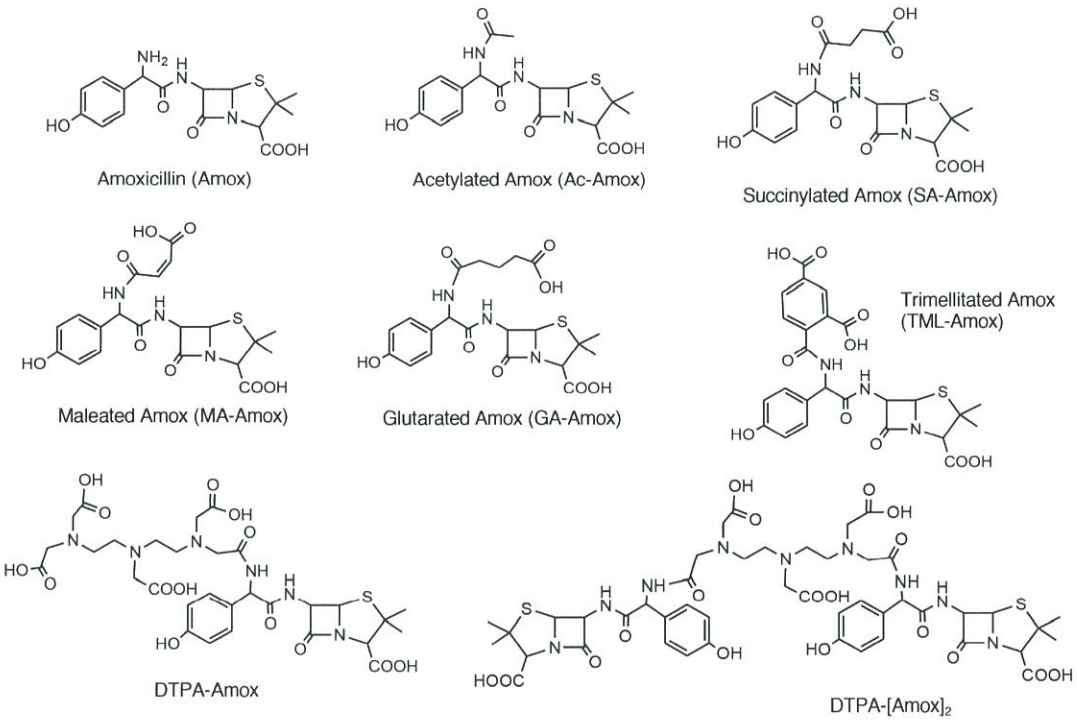


Fig 1. Development of carboxypenicillin-ureidopenicillin hybrids by introduction of carboxyl groups into the amino acid side chains of ampicillin derivatives. R2, -H (ampicillin); -OH (amoxicillin).



829

830 **Fig 2. Chemical structures of amoxicillin and its amino group-modified derivatives**

831 **synthesized in this study.**

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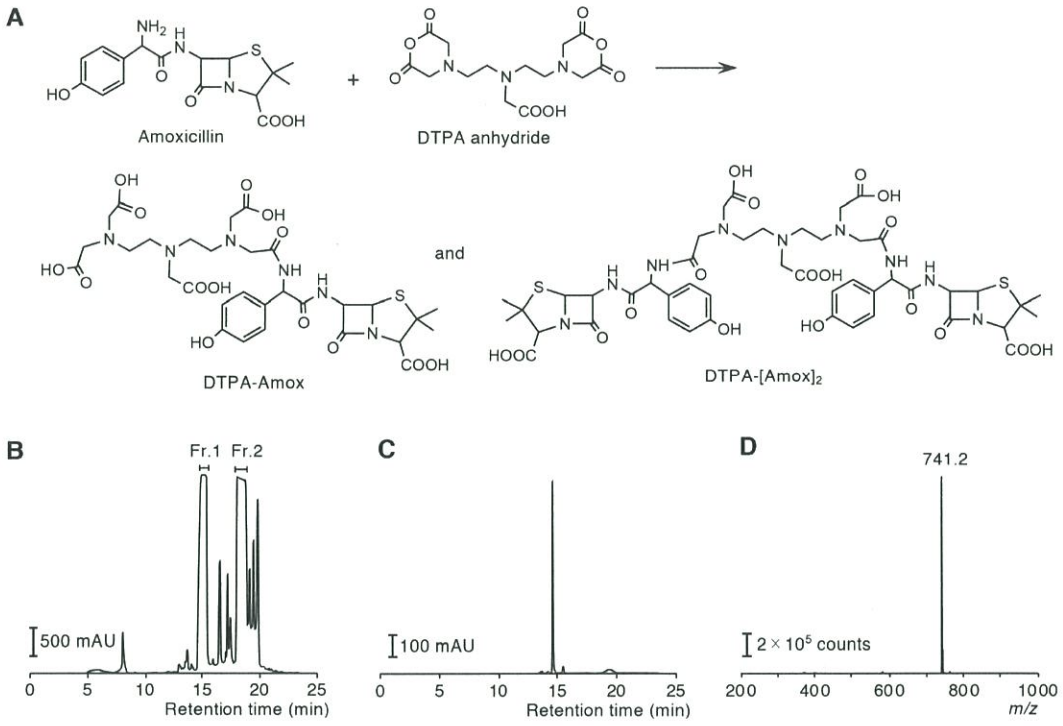

834

835 **Fig 3. Synthesis and characterization of DTPA-Amox.** A, Synthetic pathway. B,
836 Reverse-phase HPLC chromatogram for the reaction mixture of amoxicillin and DTPA
837 anhydride. DTPA-Amox and DTPA-$[Amox]_2$ were eluted in Fraction 1 (Fr. 1; DTPA-
838 Amox) and Fraction 2 (Fr. 2; DTPA-$[Amox]_2$) and were collected for lyophilization. C,
839 Reverse-phase HPLC chromatogram of purified DTPA-Amox. D, Mass chromatogram
840 of purified DTPA-Amox.

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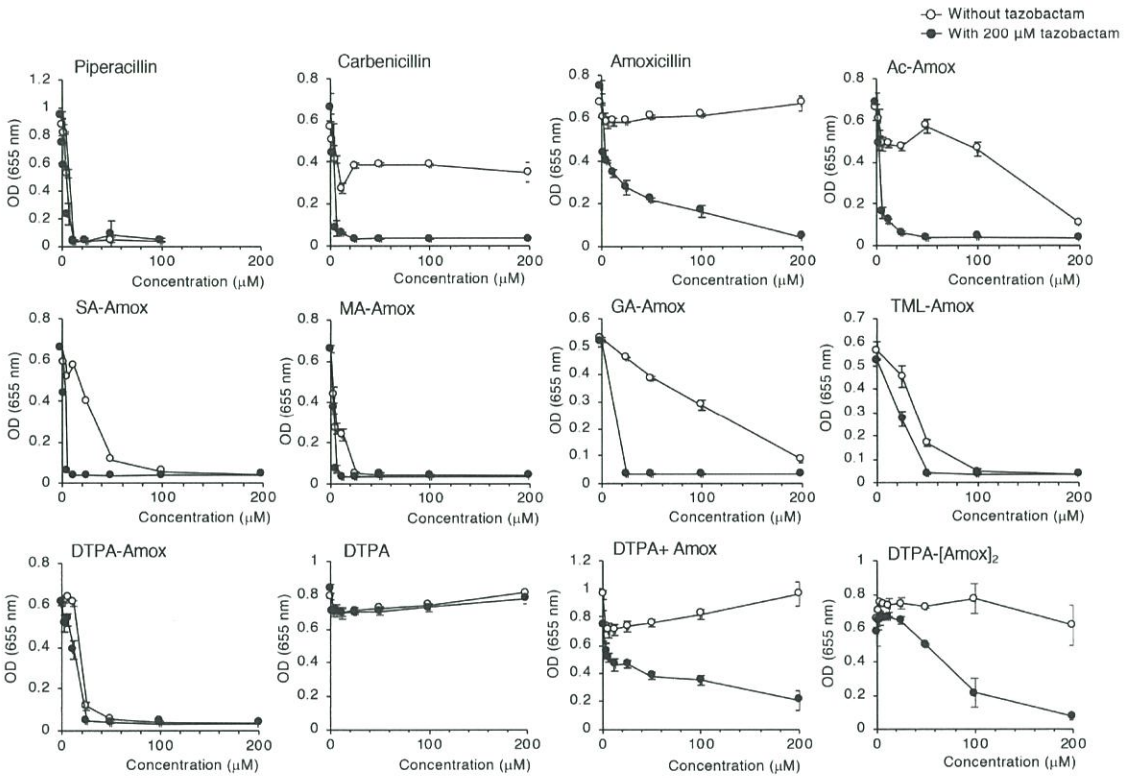
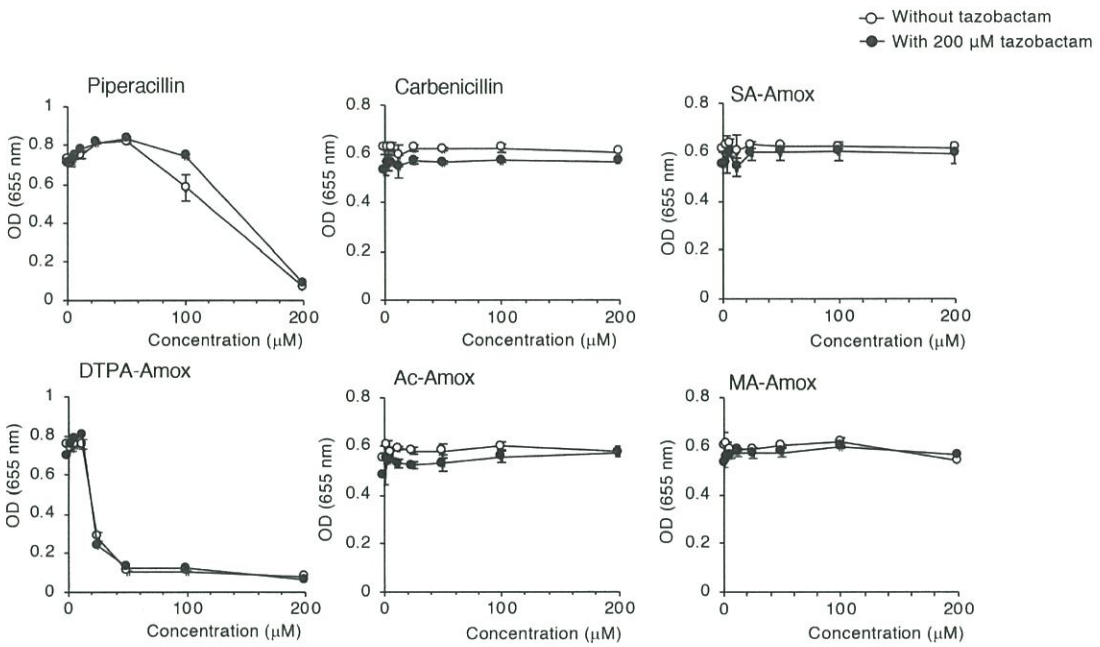


Fig 4. Antipseudomonal effects of piperacillin, carbenicillin, amoxicillin, and amino group-modified amoxicillin derivatives against *P. aeruginosa* PAO1. The *P. aeruginosa* strain PAO1 was cultured overnight at 37°C in the presence of the indicated concentrations of antibiotics in LB medium with the pH adjusted to 7.4. Bacterial growth was determined by measuring turbidity at 655 nm absorbance. Data are means ± SD ($n = 3$).



852

853 **Fig. 5 Antipseudomonal effects of piperacillin, carbenicillin, and amoxicillin**
854 **derivatives synthesized in this study against *P. aeruginosa* clinical isolate MR13**
855 **strain.** The MR13 *P. aeruginosa* clinical isolate was cultured overnight at 37°C in the
856 presence of the indicated concentrations of antibiotics in LB medium, pH 7.4. Bacterial
857 growth was determined by measuring turbidity at 655 nm absorbance. Data are means ±
858 SD (n = 3).

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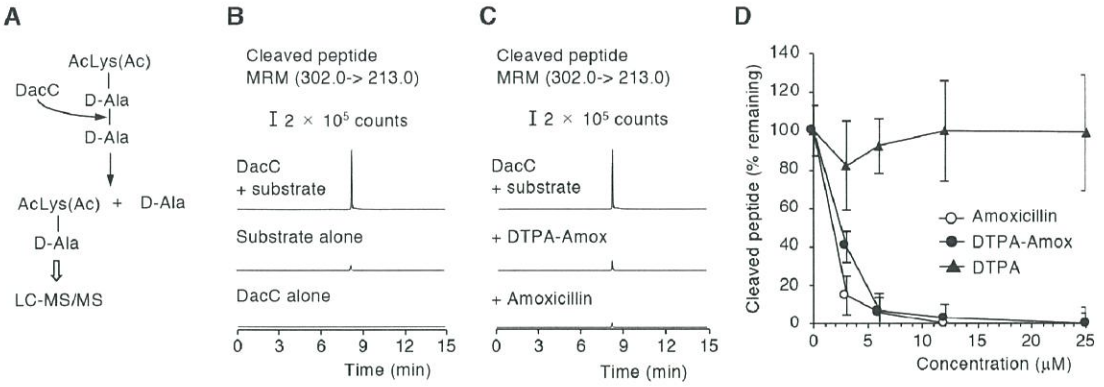
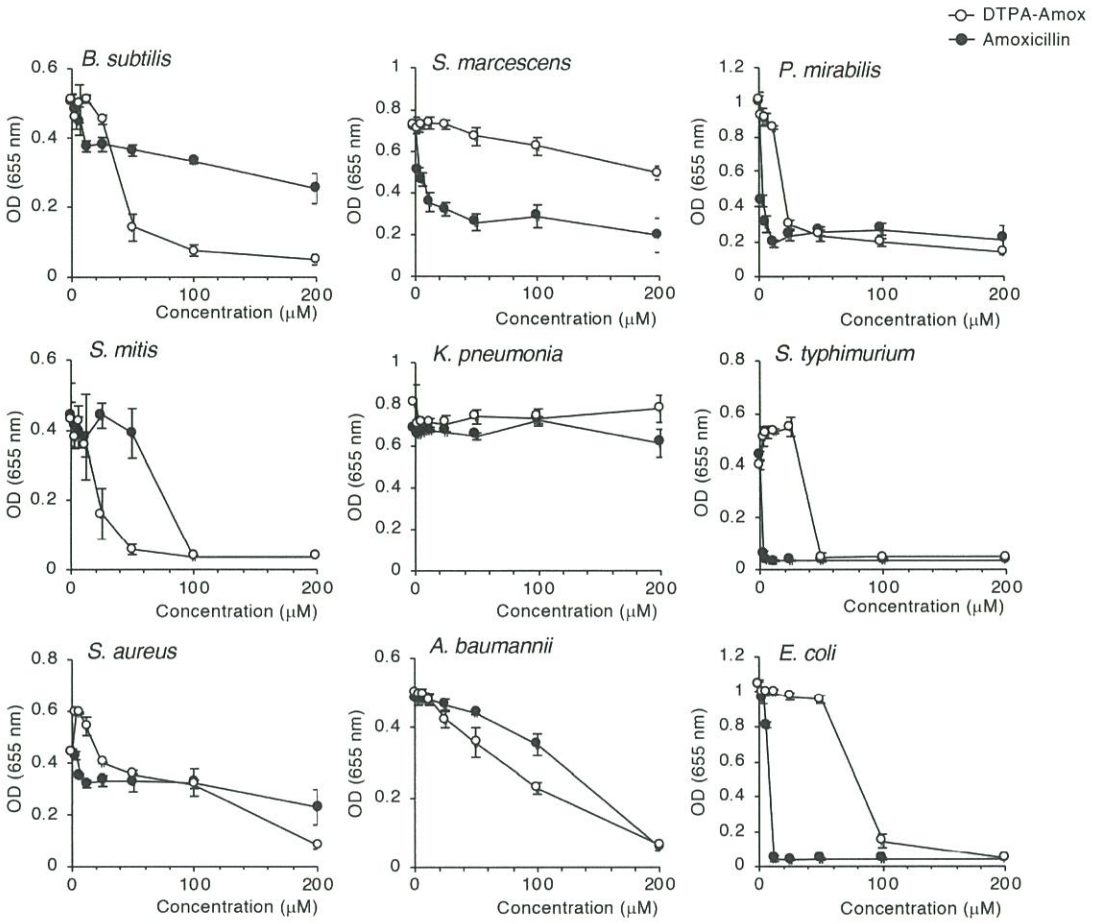


Fig 6. Inhibitory effects of DTPA-Amox on the carboxypeptidase activity of DacC. A, DacC-mediated cleavage of the D-Ala-D-Ala-containing peptide. The cleaved peptide can be quantitated by means of LC-MS/MS. Multiple reaction monitoring (MRM) of cleaved peptides derived from the DacC reaction (B,C). D, Effects of amoxicillin, DTPA-Amox, and DTPA on DacC-mediated cleavage of synthetic peptide. The DacC reaction was carried out in the presence of the indicated concentrations of additives. Data are means ± SD ($n = 3$).



871

872 **Fig 7. Antibacterial spectra of amoxicillin and DTPA-Amox**. Bacteria were cultured
873 overnight at 37°C in the presence of the indicated concentrations of amoxicillin or
874 DTPA-Amox, in pH-adjusted media. Bacterial growth was determined by measuring
875 turbidity at 655 nm absorbance. Data are means ± SD (n = 3).

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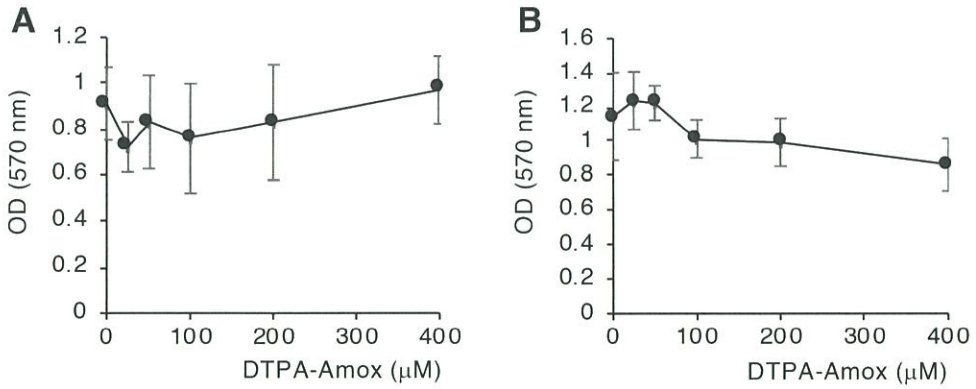

878

879 **Fig. 8. Cytotoxicity of DTPA-Amox.** Viability of HeLa cells (A) and C26 cells (B)
880 treated overnight with the indicated concentrations of DTPA-Amox. The MTT assay
881 was utilized to determine viability. Data are means ± SD (n = 3).

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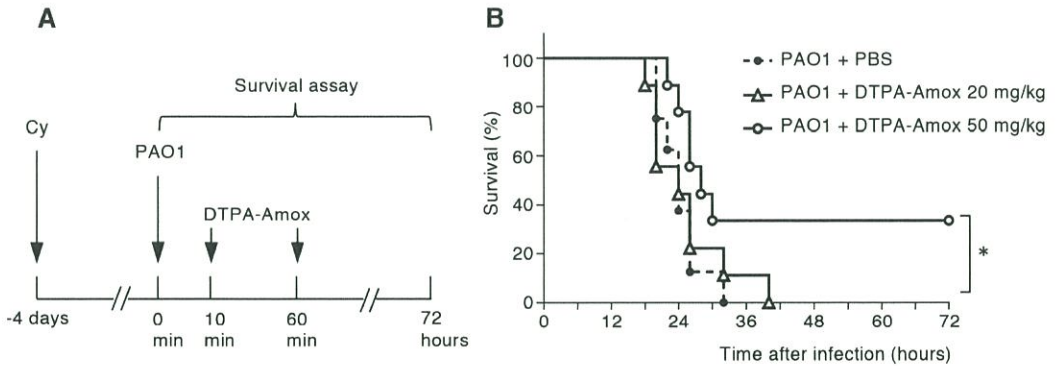

884

885 **Fig. 9. Therapeutic effect of DTPA-Amox on *P. aeruginosa* infection in a mouse**
886 **model of leukopenia.** A, Time line of the *in vivo* treatment. B, Mice received
887 intraperitoneal injections of cyclophosphamide (Cy; 250 mg/kg) and were then infected
888 with *P. aeruginosa* strain PAO1 (5×10^5 CFU). At 10 and 60 min after infection, mice
889 received intraperitoneal injections of PBS (controls), 20 mg/kg DTPA-Amox, or 50
890 mg/kg DTPA-Amox. Survival of the mice was monitored for 72 hours after the
891 infection. PAO1 + PBS group $n = 8$; PAO1 + DTPA-Amox treatment groups $n = 9$. *p
892 < 0.05.

893

Supplementary figures for

Development of potent antipseudomonal β-lactams by means of polycarboxylation of aminopenicillins

Short title: Polycarboxylated aminopenicillins as potent antipseudomonal agents

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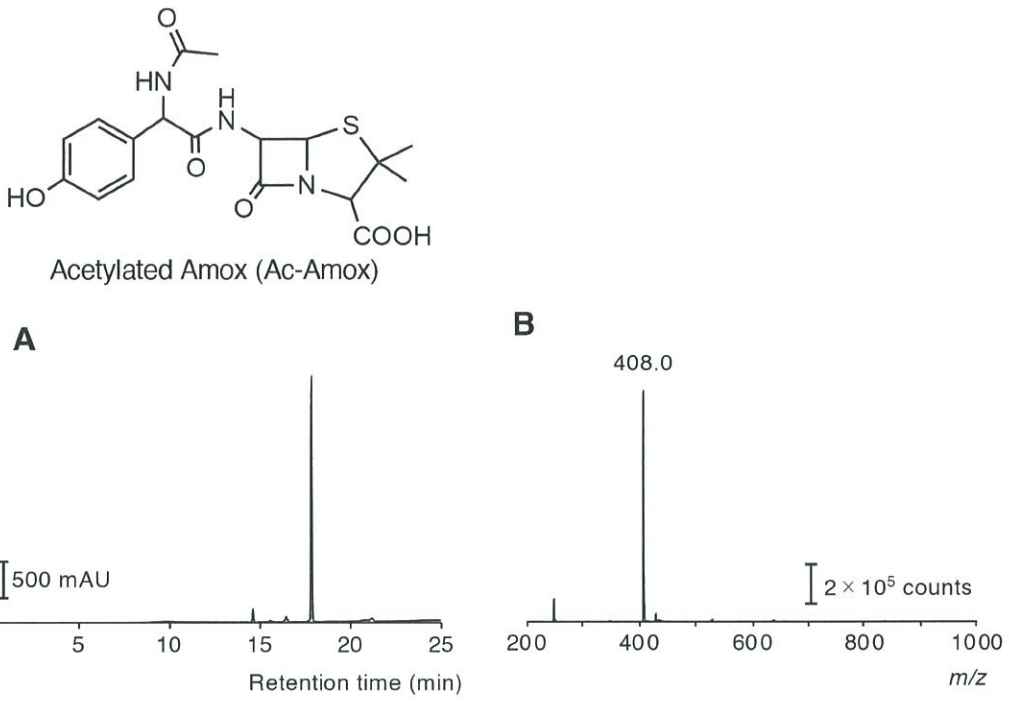
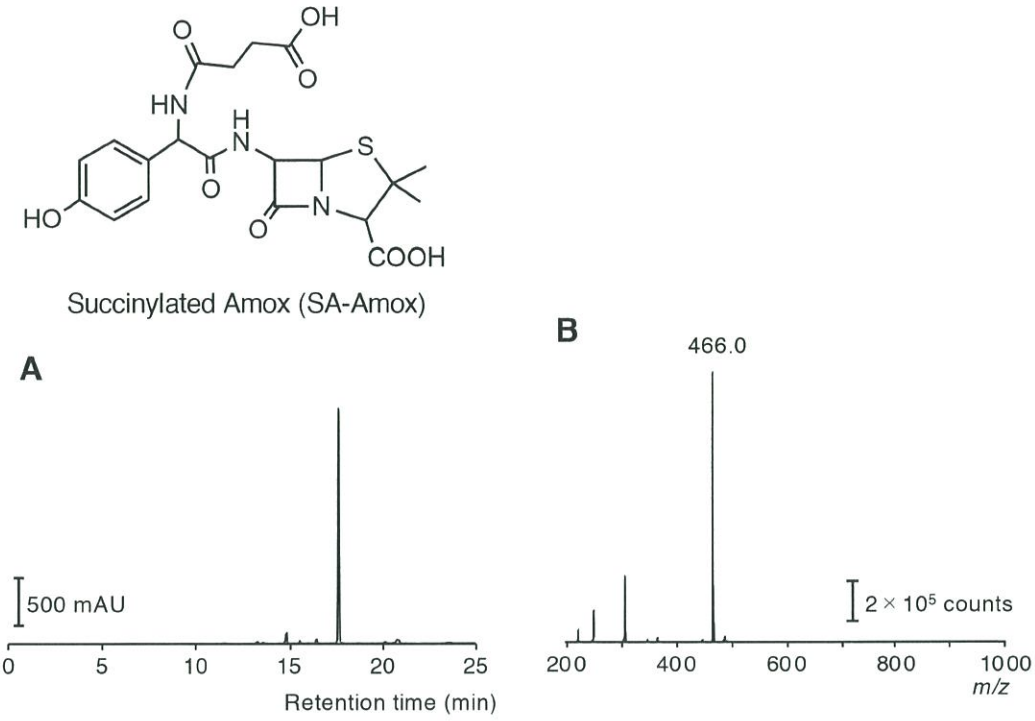
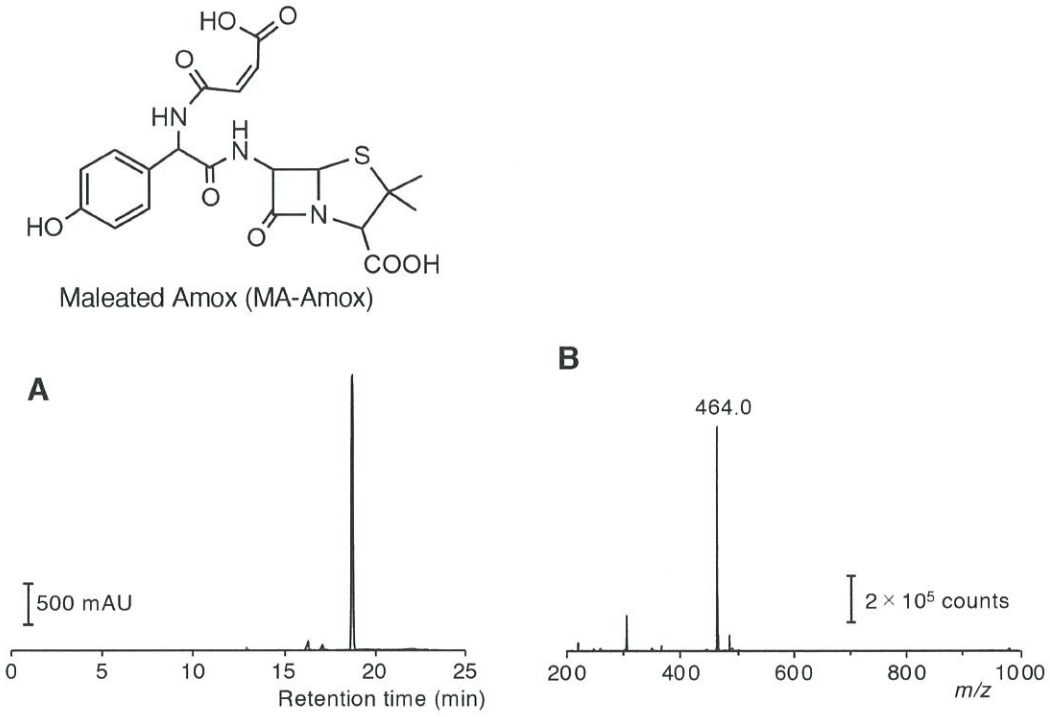


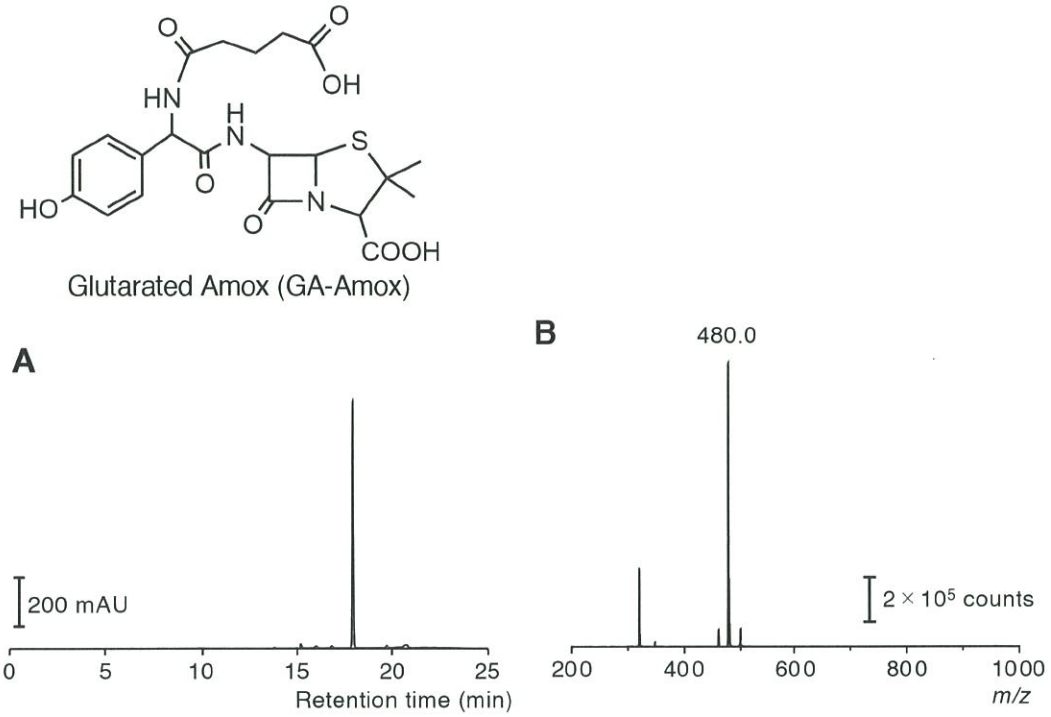
S1 Fig. Characterization of Ac-Amox. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



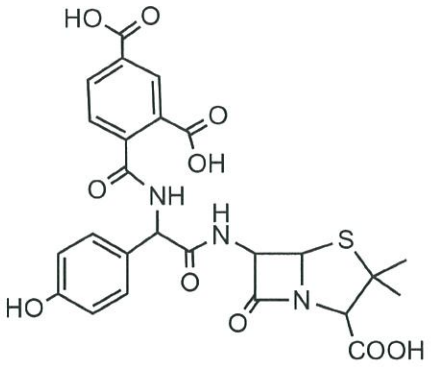
S2 Fig. Characterization of SA-Amox. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



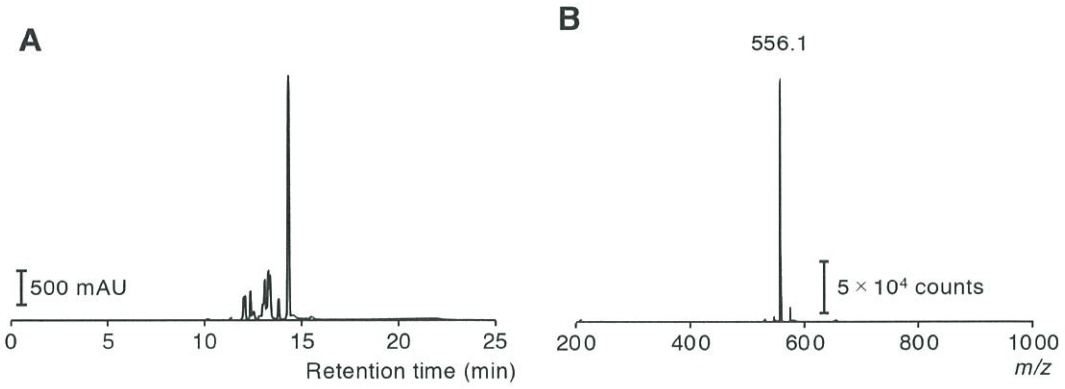
S3 Fig. Characterization of MA-Amox. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



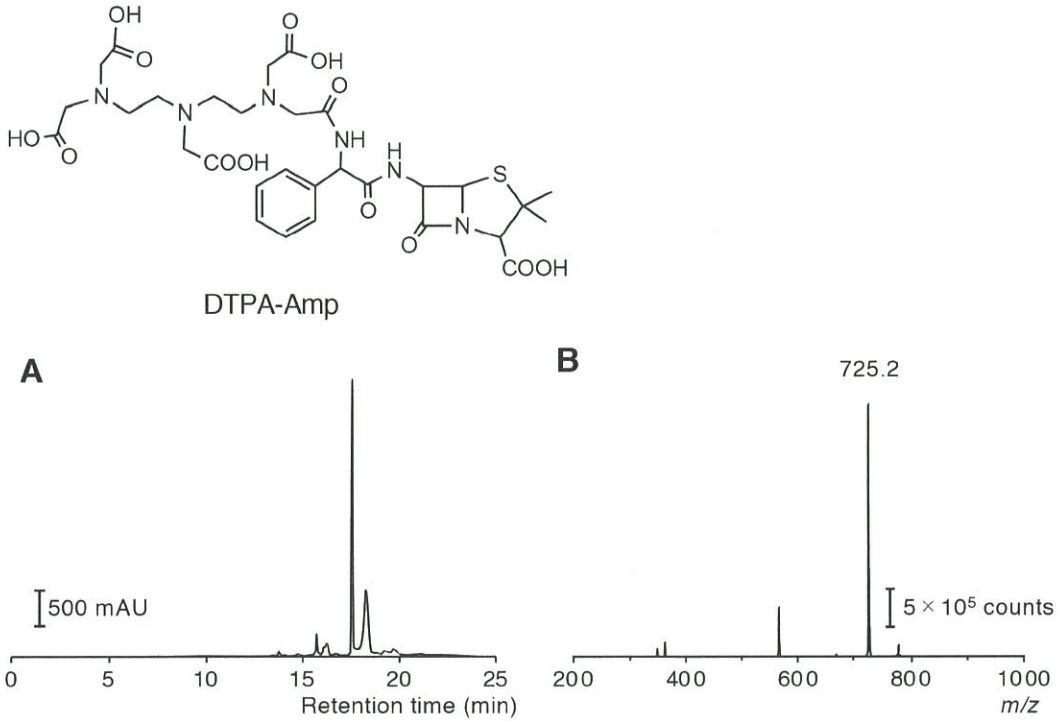
S4 Fig. Characterization of glutarated Amox (GA-Amox). A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



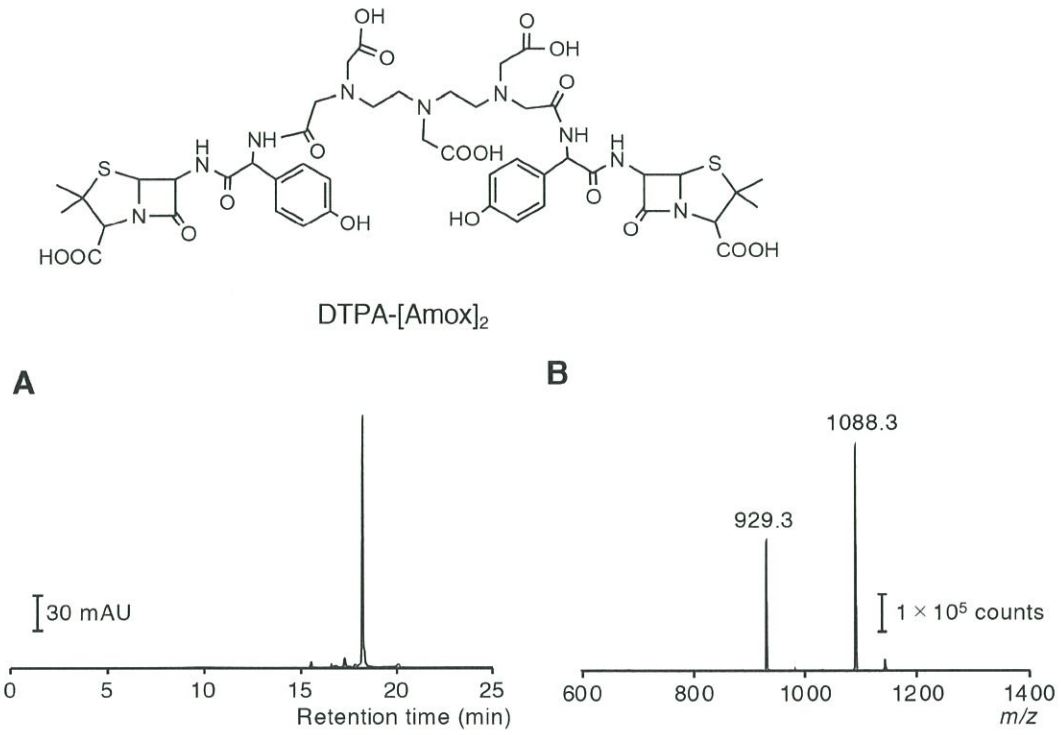
Trimellitated Amox (TML-Amox)



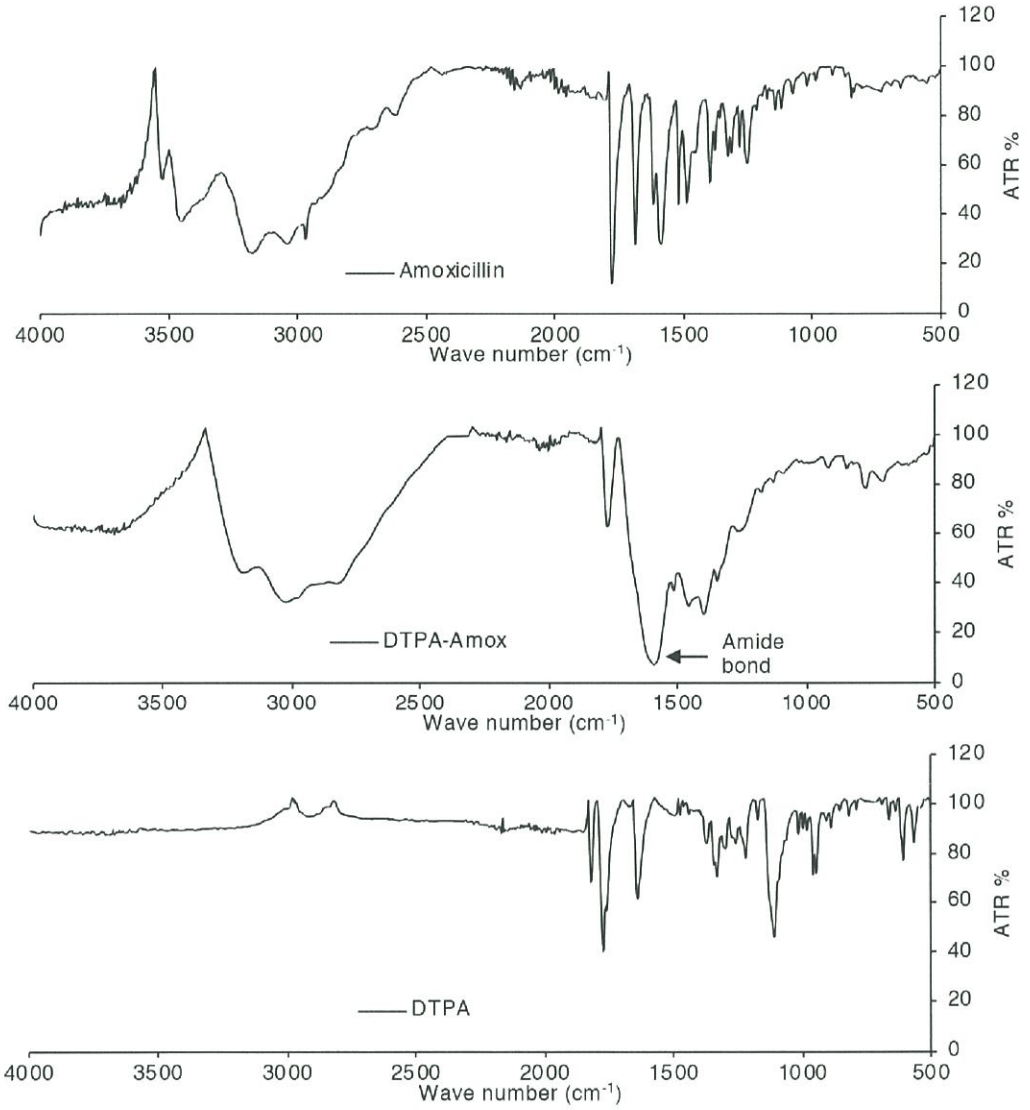
S5 Fig. Characterization of trimellitated Amox (TML-Amox). A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



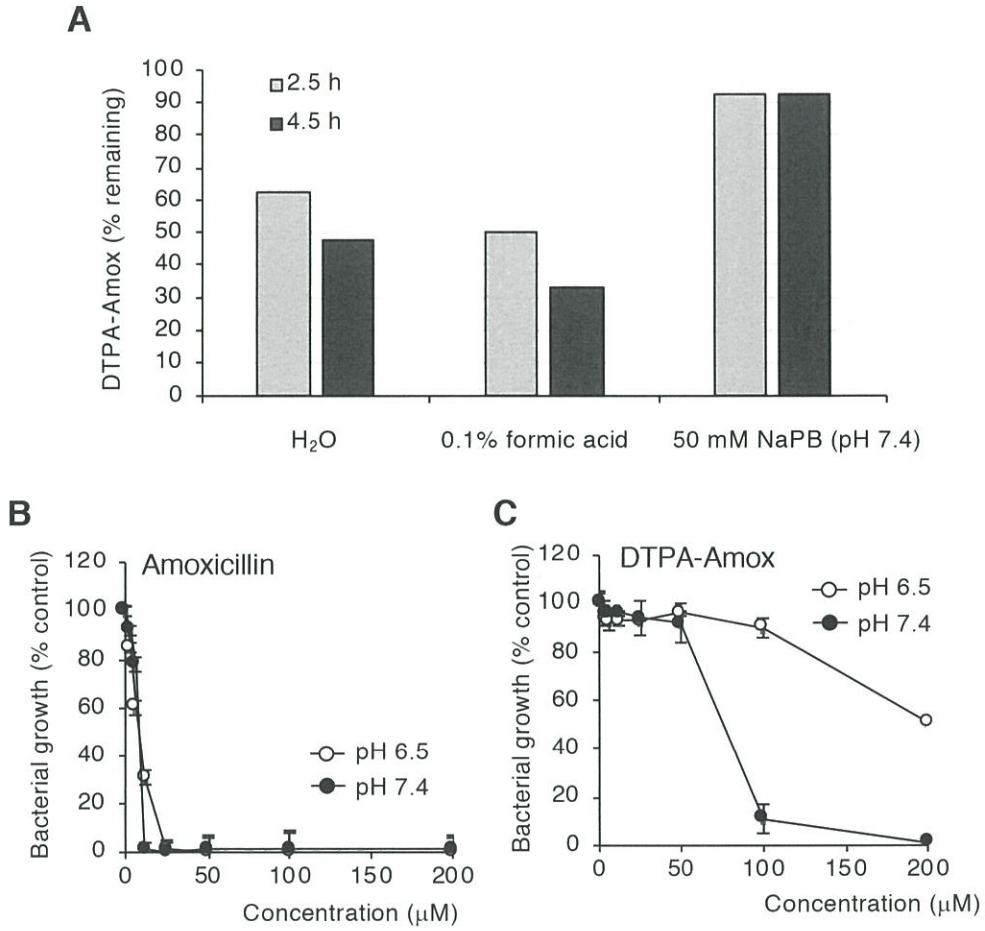
S6 Fig. Characterization of DTPA-conjugated ampicillin **(DTPA-Amp).** A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



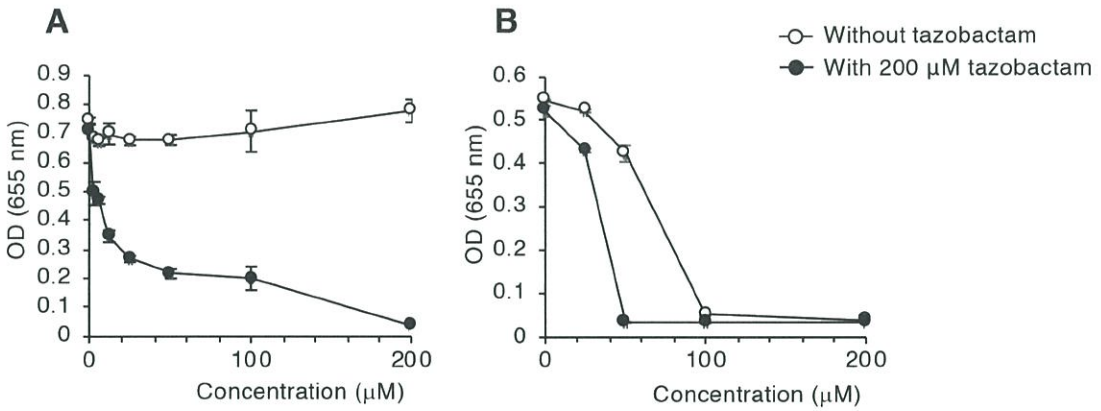
S7 Fig. Characterization of DTPA-[Amox]$_2$. A, Reverse-phase HPLC chromatogram. B, Mass chromatogram.



S8 Fig. Infrared spectra of amoxicillin, DTPA-Amox, and DTPA.



S9 Fig. Effects of culture medium pH on the stability and antibacterial activities of amoxicillin and DTPA-Amox. A, Stability of DTPA-Amox in different media. DTPA-Amox was dissolved in H_2O, 0.1% formic acid, or 50 mM sodium phosphate buffer (pH 7.4), followed by incubation at 37°C for 2.5 or 4.5 hours. DTPA-Amox remaining in the medium was quantitated by means of HPLC. Antibacterial activities of native amoxicillin (B) and DTPA-Amox (C) against *E. coli*. LB medium was used without pH adjustment (pH 6.5) or with pH adjusted with sodium phosphate buffer (NaPB) to pH 7.4. Bacterial growth was determined by measuring turbidity at 655 nm absorbance after overnight culture. Data are means ± SD ($n = 3$).



S10 Fig. Antipseudomonal effects of (A) ampicillin and (B) DTPA-conjugated ampicillin**.** *P. aeruginosa* strain PAO1 was cultured overnight at 37°C in the presence of the indicated concentrations of antibiotics in LB medium at pH 7.4. Bacterial growth was determined by measuring turbidity at 655 nm absorbance. Data are means ± SD ($n = 3$).