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RESEARCH ARTICLE

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Synthesis and antimicrobial activity of the hybrid molecules between amoxicillin and derivatives of benzoic acid

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Abstract

Due to the increasing problem of bacterial resistance worldwide, the demand for new antibiotics is becoming increasingly urgent. We wished to: (a) prepare hybrid molecules by linking different pharmacophores by chemical bonds; (b) investigate the antib acterial activity of these hybrids using drug-sensitive and drug-resistant pathogens in vitro and *vivo*. A series of hybrid molecules with a diester structure were designed and synthesized that linked amoxicillin and derivatives of benzoic acid via a methylene bridge. Synthesized compounds were evaluated for activities against Gram-positive bacteria (*Staphylococcus aureus* American Type Culture Collection [ATCC] 29213, ATCC 11632; methicillin-resistant *S. aureus* [MRSA] 11; *Escherichia coli* ATCC 25922) and Gram-negative bacteria (*Salmonella* LS677, GD836, GD828, GD3625) by microdilution of broth. Synthesized compounds showed good activity against Gram-positive and Gram-negative bacteria in vitro. In particular, amoxicillin-*p*-nitrobenzoic acid (**6d**) showed good activity against *Salmonella* species and had better activity against methicillin-resistant *S. aureus* (minimum inhibitory concentration [MIC] = 64 μg/ml) than the reference drug, amoxicillin (MIC = 128 μg/ml). Amoxicillin-*p*-methoxybenzoic acid (**6b**) had the best antibacterial activity in vivo (ED_{50} = 13.2496 μg/ml). The hybrid molecules of amoxicillin and derivatives of benzoic acid synthesized based on a diester structure can improve the activity of amoxicillin against *Salmonella* species and even improve the activity against MRSA.

KEYWORDS

amoxicillin, antibacterial activity, benzoic-acid derivative, drug design, hybrid molecule

1 | INTRODUCTION

The abuse of antibiotics in intensive farming in developing countries can: (a) result in the residue of antibiotics in animal-derived products; (b) increase the number of antibiotic-resistant bacteria in the gut of animals (Christy, Sampson, Edson, & Anthony, 2018; Sørum & Sunde, 2001). The emergence of multi-drug resistant bacteria, especially Gram-positive bacteria (e.g., penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant enterococci) poses a major threat to public health (Bennett, 2010; Goossens & Herman, 2005; Lim & Webb, 2005).

The growing problem of bacterial resistance has spurred our interest in discovering new and more efficacious antimicrobial agents to address the problem of animal-borne bacterial resistance at the source (Chauhan, Hati, Priyadarshini, & Sen, 2019). However, due to the long research cycle of traditional methods of new-drug development and the expense of research, scholars are beginning to seek new tools for drug development (Dickson & Gagnon, 2004; Dimasi, Hansen, & Grabowski, 2003). The concept of pharmacophore

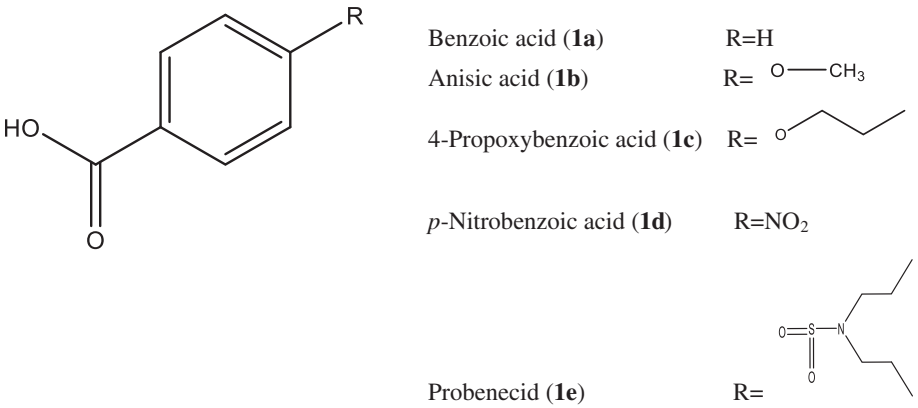


FIGURE 1 Benzoic acid and its derivatives

hybridization has attracted increasing interest from pharmaceutical chemists. According to the report, a rational drug design method based on the synthesis of a double ligand or a multi-ligand can produce a drug with better clinical effects than a single targeted drug. (Mandal, Moudgil, & Mandal, 2009). Moreover, certain hybrid molecules exhibit better pharmacological advantages and lower toxicity than simple combination formulations.(Catane, Kaufman, Madajewicz, Mittelman, & Murphy, 1978; English, Girard, & Haskell, 1984).

Currently, β-lactam antibiotics (BLAs) are the most important anti-infective drugs used in the clinical treatment of animals. The semi-synthetic penicillin amoxicillin was developed in the 1960s. The spectrum and effect of amoxicillin against most bacteria in vitro are basically identical to those of ampicillin. However, the bactericidal effect of amoxicillin against various bacteria is faster and stronger than that of ampicillin. Amoxicillin has been used widely in veterinary clinics because of its stability, rapid absorption in the body, strong bactericidal power, few side-effects, diverse preparations, convenient combination with other drugs, and low price.

Studies have shown that BLAs inhibit the formation of bacterial cell walls. The target molecules for the action of BLAs on bacterial cell walls are penicillin-binding proteins because of the stereochemical similarity between β-lactam and D-alanine-dialanine substrates (Yocum, Waxman, Rasmussen, & Strominger, 1979). In this way, a BLA can form a lethal covalent penicillin–enzyme complex with a transpeptidase, which is used to block normal transpeptidation in bacteria (Wilke, Lovering, & Strynadka, 2005). Pharmacodynamic studies have shown that BLAs are time-dependent antibacterial drugs, and that the key to enhancing their efficacy is to maximize the time when the plasma concentration is greater than the minimum inhibitory concentration (MIC) (Hohlfelder, Kubiak, Degrado, Reardon, & Szumita, 2016).

Benzoic acid is widely found in nature in the form of free acids, esters, or derivatives thereof. It has been well documented that various derivatives of benzoic acid have great potential for antibacterial, antiviral, and anti-inflammatory effects (Inglis et al., 2007; Sapra, Kumar, Kakkar, & Narasimhan, 2014; Selvam, Breitenbach, Borysko, & Drach, 2009). In the 1940s, it was reported that benzoic acid (Figure 1) increased the blood level of penicillin by 4–8-fold, and that the time benzoic acid remained in the blood was extended (Bronfenbrenner & Favour, 1945). In recent years, studies have shown that probenecid can increase the serum concentration and prolong the duration of action of BLAs such as penicillin by inhibiting the secretion of these weak organic acids in the proximal and distal tubules of the kidney. Probenecid is a derivative of benzoic acid used to treat chronic gout, and acts by competitively inhibiting renal-tubular reabsorption of uric acid and increasing its excretion (Brown, Zemcov, & Clarke, 1993; Overbosch, Gulpen, Hermans, & Mattie, 1988; Tanizaki et al., 2015).

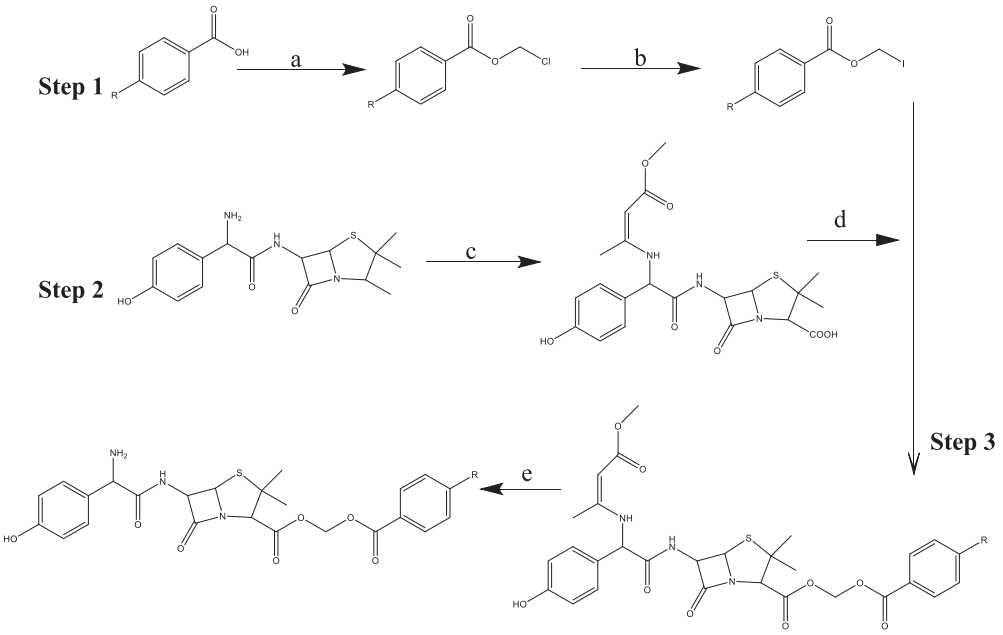
To investigate the antibacterial activity and pharmacokinetic characteristics of the heterozygous molecules of amoxicillin and derivatives of benzoic acid (Figure 1), we linked amoxicillin and benzoic-acid derivatives via a methylene bridge. The synthetic route is shown in Scheme 1. We also investigated the antibacterial activity of hybrids using drug-sensitive and drug-resistant pathogens by standard methods in vitro. Results indicated that these molecules had excellent properties against Gram-negative bacteria such as *Salmonella* species. Also, compound **6d** had good activity against MRSA.

2 | MATERIALS AND METHODS

2.1 | Chemicals and instruments

The starting materials, reagents, and solvents used in the present study were purchased from J & K Chemicals (Beijing, China) or Sigma–Aldrich (Saint Louis, MO). Infrared (IR) spectroscopy was carried out on an Affinity-1 spectrometer (Shimadzu, Kyoto, Japan) using potassium-bromide disks. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was undertaken on a spectrometer (AVANCE AV600; Bruker Billerica, MA) in dimethylsulfoxide (DMSO). Mass spectroscopy (MS) was carried out with an Orbitrap™ Elite system (Thermo Fisher Scientific, Waltham, MA) using the electrospray ionization (ESI) method.

The preparation of target compounds is outlined in Scheme 1. Identification of the structures of the synthesized compounds was supported by data from IR, ^{1}H NMR, and ^{13}C NMR spectroscopy. The results of HR-MS (ESI) were in agreement with the proposed structures.



SCHEME 1 Synthesis of hybrid molecules between amoxicillin and derivatives of benzoic acid. Reagents and conditions: (a) Chloroiodomethane, triethylamine, DMF, room temperature, stirring for 5 hr. (b) NaI, acetone aqueous solution, room temperature, stirring in the dark for 18 hr. (c) Methyl acetoacetate, DMF, K_2CO_3, stirring for 2 hr at room temperature followed by another 4 hr at 0 °C. (d) DMF, 0–5 °C, stirring for 4 hr. (e) Acetone aqueous solution, 10% HCl, stirring for 0.5 hr

2.2 | General procedure for the synthesis of benzoic chloromethyl ester (2a–e)

To a solution of **1a** (7.32 g, 60 mmol) in dimethylformamide (50 ml) was added triethylamine (8 ml, 58 mmol) and chloroiodomethane (28 ml, 0.38 mol). The mixture was stirred for 5 hr at room temperature. After dilution with ethyl acetate (100 ml), the mixture was washed with water (3 × 100 ml) and then by brine (100 ml). The mixture was dried and evaporated in vacuo to leave the desired compound: a yellowish oil. The residue underwent chromatography using petroleum ether/ethyl acetate (4:1, v:v).

Chloromethyl benzoate (2a) White solid; yield: 45.42%; Rf: 0.75; IR (KBr, cm^{-1}): 2,987.74, 1,741.72 (C=O), 1,597.06, 1,444.68, 1,342.46, 1,255.66, 1,174.65, 1,078.21; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.10–8.06 (d, 2H), 7.61 (t, J = 7.4, 1.4 Hz, 1H), 7.46 (t, J = 7.8 Hz, 2H), 5.96 (s, 2H, CH_2Cl); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 164.54, 133.94, 130.06, 128.62, 69.30.

HR-MS (ESI) Calcd for $C_8H_7ClO_2$ ($M + H^+$): 171.5273; Found: 171.5276; purity:97.85% (determined by HPLC).

Chloromethyl 4-methoxybenzoate (2b) White solid; yield: 45.42%; Rf: 0.71; IR (KBr, cm^{-1}): 2,970.38, 2,845.00, 1,734.01 (C=O), 1,606.70, 1,510.26, 1,448.54, 1,334.74, 1,259.52, 1,172.72, 1,082.07, 1,024.20; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.04–8.00 (d, 2H), 6.92–6.90 (d, 2H), 5.93 (s, 2H, CH_2Cl), 3.83 (s, 3H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 164.19, 132.24, 120.92, 113.89, 69.22, 55.51.

HR-MS (ESI) Calcd for $C_9H_9ClO_3$ ($M + H^+$): 201.5408; Found: 200.5411; purity:98.64% (determined by HPLC).

Chloromethyl 4-ethoxybenzoate (2c) White solid; yield: 46.63%; Rf: 0.65; IR (KBr, cm^{-1}): 2,968.45, 2,877.79, 1,735.93 (C=O), 1,606.70, 1,510.26, 1,438.90, 1,332.81, 1,257.59, 1,168.86, 1,080.14, 1,010.70; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.03 (d, 2H), ^{1}H NMR (600 MHz, Chloroform-d) δ 8.03 (d, J = 8.2 Hz, 2H), 6.94 (d, J = 8.2 Hz, 2H), 5.95 (s, 2H, CH_2Cl), 3.99 (s, 2H), 1.85 (q, J = 7.1 Hz, 2H), 1.06 (t, J = 7.4 Hz, 3H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 164.24, 163.81, 132.22, 120.64, 114.34, 69.79, 69.20, 22.41, 10.42.

HR-MS (ESI) Calcd for $C_{11}H_{13}ClO_3$ ($M + H^+$): 229.6723; Found: 229.6726; purity:97.51% (determined by HPLC)550.

Chloromethyl 4-nitrobenzoate (2d) was prepared according to step 1. White solid; yield: 36.74%; Rf: 0.77; IR (KBr, cm^{-1}): 3,005.10 (benzene ring), 1,737.86, 1,604.77, 1,525.69, 1,433.11, 1,338.60, 1,253.73, 1,082.07, 1,010.70; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.35–8.27 (dd, 4H), 6.00 (s, 2H, CH_2Cl); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 162.86, 151.09, 133.93, 131.21, 123.74, 69.52.

HR-MS (ESI) Calcd for $C_8H_6ClNO_4$ ($M + H^+$): 216.5890; Found: 216.5887; purity:98.32% (determined by HPLC).

Chloromethyl 4-(*N*,*N*-dipropylsulfamoyl)benzoate (2e) was prepared according to step 1. White solid; yield: 32.52%; Rf: 0.0.72; IR (KBr, cm^{-1}): 2,972.31, 2,935.66, 2,873.94, 1,747.51 (C=O), 1,597.06, 1,450.47, 1,394.53, 1,342.46, 1,251.80, 1,157.29, 1,078.21; ^{1}H NMR (600 MHz, Chloroform-d) δ 8.19 (d, J = 8.6 Hz, 2H), 7.90 (d, J = 8.6 Hz, 2H), 5.97 (s, 2H, CH_2Cl), 3.13–3.07 (m, 4H), 1.58–1.51 (m, 4H), 0.86 (t, J = 7.4 Hz, 6H). (t, J = 7.4 Hz, 6H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 163.34, 145.29, 131.82, 130.67, 127.15, 69.44,

49.91, 21.91, 11.12.

HR-MS (ESI) Calcd for $C_{14}H_{20}ClNO_4S$ (M + H^+): 334.8270; Found: 334.8276.

2.3 | Synthesis of (*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (3)

To a suspension of potassium carbonate (7.6 g, 55 mM) in 10 ml of dimethylformamide was added 10.8 ml (100 mM) of methyl acetoacetate and 20.97 g (50 mM) of amoxicillin trihydrate. The reaction mixture was stirred for 2 hr at room temperature followed by another 4 hr at 0 °C. The solvent was decanted and the residue washed with 25 ml of diethyl ether. The residue was dissolved in 15 ml of acetone and the insoluble material filtered through celite. The filtrate was diluted with 15 ml of isopropanol and evaporated in vacuo to remove acetone. Recrystallization was induced by scratching, and the resulting suspension was maintained for 12 hr at 0 °C. The residue underwent chromatography using ethyl acetate/methanol (3:2, v:v). And the precipitated solid was washed with cold isopropanol and diethyl ether to give a fluffy white solid.

(*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (**3**): White solid; yield: 86.98%; Rf: 0.76; IR (KBr, cm^{-1}): 3,302.13 (OH), 2,964.59, 1,772.58 (C=O), 1,664.57, 1,597.06, 1,508.33, 1,446.61, 1,392.61, 1,269.16, 1,172.72, 1,134.14; ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.70 (s, 1H, COOH), 9.25 (d, J = 8.3 Hz, 1H,CONH), 8.86 (d, J = 8.5 Hz, 1H, NH), 7.13 (d, J = 8.1 Hz, 2H), 6.75 (d, J = 8.2 Hz, 2H), 5.48–5.44 (m, 1H), 5.42–5.37 (m, 1H), 5.30–5.26 (m, 1H), 4.45 (s, 1H), 3.88 (s, 1H), 3.52 (s, 3H), 1.76 (s, 3H), 1.56 (s, 3H), 1.42 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ:173.43, 170.67, 169.93, 169.83, 160.65, 158.30, 128.91, 127.79, 116.00, 83.41, 74.63, 66.94, 64.87, 58.23, 57.57, 50.01, 31.63, 27.96, 19.66.

HR-MS (ESI) Calcd for $C_{21}H_{25}N_3O_7S$ (M + H^+): 464.5050; Found: 464.5057; purity:97.41% (determined by HPLC).

2.4 | General procedure for the synthesis of the hybrid molecules between amoxicillin and the derivatives of benzoic acid (5a–e, 6a–e)

Anhydrous sodium iodide (3.1 g, 32 mmol) was added to EtOAc (15 ml) and stirred for 16 hr at room temperature. Then, the temperature of the system was lowered to 0–5 °C, the pH was adjusted to 3.0–7.3 with sodium hydrogen carbonate under stirring, and the solution decolorized by dropwise addition of a 0.1 mol/L sodium thiosulfate solution. During stirring, 15 ml of water was added dropwise to the solution to precipitate white crystals. and the residue was washed with an aqueous acetone solution (30 ml; 1:1, v:v) to give compounds **4a–e**. Then, compound **4a–e** (0.1 mol) was condensed with the carboxylate ion of compound **2** (0.114 mol) at 5 °C to obtain compound **5a–e**. The crude product obtained underwent chromatography using petroleum ether/ethyl acetate (1:1, v:v). The condensation product was dissolved in an aqueous acetone solution. The pH of the solution was adjusted to 2.5 with 10% HCl, deprotected and, then, the acetone in the solution was removed by vacuum spinning. Next, NaCl was added to the solution until it was saturated, followed by extraction of an acetone/ethyl acetate solution (1:2, v:v). The organic layer was removed and dried under reduced pressure. Finally, the crude product was purified by column chromatography using silica gel to afford compound **6a–e**. The crude product obtained underwent chromatography using ethyl acetate/methanol (5:1, v:v).

2.4.1 | (Benzoyloxy)methyl(*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (5a)

Light-yellow solid; yield: 72.68%; Rf: 0.69; IR (KBr, cm^{-1}): 3,356.14 (OH), 3,064.89, 2,974.23, 1,778.37 (C=O), 1,666.50, 1,600.92, 1,508.33, 1,448.54, 1,269.16, 1,168.86; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.07 (d, *J* = 7.0 Hz, 1H, CONH), 8.07 (d, *J* = 7.7 Hz, 2H), 7.63 (t, *J* = 7.5 Hz, 1H), 7.48 (t, *J* = 7.7 Hz, 2H), 7.17 (d, *J* = 8.3 Hz, 2H), 6.91 (d, *J* = 9.0 Hz, 1H, NH), 6.72 (d, *J* = 8.4 Hz, 2H), 6.07 (dd, *J* = 39.7, 5.7 Hz, 2H, OCH_2O), 5.64 (dd, *J* = 8.9, 4.1 Hz, 1H), 5.57–5.54 (m, 1H), 5.03 (d, *J* = 6.9 Hz, 1H), 4.67 (s, 1H), 4.48 (s, 1H), 3.67–3.64 (m, 3H), 1.93 (s, 3H), 1.56 (s, 3H), 1.46 (s, 3H); ^{13}C NMR (151 MHz, Chloroform-d) δ 172.89 (C=O), 170.95 (C=O), 170.70 (C=O), 166.43 (C=O), 164.90 (C=O), 160.11, 156.70, 134.00, 130.04, 128.64, 128.36, 116.37, 85.94, 80.03 (OCH_2O), 70.11, 68.01, 61.20, 60.41, 58.81, 50.47, 26.72, 21.03, 19.78.

HR-MS (ESI) Calcd for $C_{29}H_{31}N_3O_9S$ (M + H^+): 598.6390; Found: 598.6393; purity: 98.56% (determined by HPLC).

2.4.2 | ([4-Methoxybenzoyl]oxy)methyl (*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (5b)

Light-yellow solid; yield: 68.96%; Rf: 0.54; IR (KBr, cm^{-1}): 3,363.86 (OH), 2,972.31, 2,368.59, 1,782.23 (C=O), 1,604.77, 1,512.19, 1,446.61, 1,265.30, 1,170.79, 1,091.71, 1,022.27; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.09 (d, *J* = 7.0 Hz, 1H, CONH), 8.02 (d, *J* = 8.5 Hz, 2H), 7.19 (d, *J* = 8.1 Hz, 2H), 6.95 (d, *J* = 8.7 Hz, 2H), 6.87 (d, *J* = 9.2 Hz, 1H, NH), 6.74 (d, *J* = 8.1 Hz, 2H), 6.09–6.00 (m, 2H, OCH_2O), 5.64 (dd, *J* = 9.1, 4.2 Hz, 1H), 5.55 (d, *J* = 4.0 Hz, 1H), 5.03 (d, *J* = 6.9 Hz, 1H), 4.67 (s, 1H), 4.48 (d, *J* = 1.3 Hz, 1H), 3.89 (d, *J* = 1.3 Hz, 3H), 3.65 (s, 3H), 1.94 (s, 3H), 1.55 (s, 3H), 1.46 (s, 3H); ^{13}C NMR (151 MHz, Chloroform-d) δ 172.84 (C=O), 170.92 (C=O),

170.62 (C=O), 166.48 (C=O), 164.54 (C=O), 164.24, 160.05, 156.61, 132.23, 128.39, 128.19, 120.64, 116.35, 113.95, 85.98, 79.87 (OCH_2O), 70.12, 68.01, 64.87, 61.21, 55.52, 50.45, 31.58, 26.71, 19.78.

HR-MS (ESI) Calcd for $C_{30}H_{33}N_3O_{10}S$ ($M + H^+$): 628.6650; Found: 628.6646; purity:98.69% (determined by HPLC).

2.4.3 | ([4-Ethoxybenzoyl]oxy)methyl (*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (5c)

Light-yellow solid; yield: 73.56%; Rf: 0.0.76; IR (KBr, cm^{-1}): 3,356.14 (OH), 2,968.45, 1,780.30 (C=O), 1,600.92, 1,512.19, 1,444.68, 1,265.30, 1,166.93, 1,085.92; ^{1}H NMR (600 MHz, Chloroform-d) δ 9.08 (d, J = 6.9 Hz, 1H, CONH), 8.00 (d, J = 7.6 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H), 6.93 (d, J = 8.7 Hz, 2H), 6.88 (d, J = 9.0 Hz, 1H, NH), 6.74 (d, J = 7.5 Hz, 2H), 6.04 (dd, J = 39.5, 5.6 Hz, 2H, OCH_2O), 5.64 (dd, J = 8.8, 4.0 Hz, 1H), 5.55 (d, J = 4.1 Hz, 1H), 5.03 (d, J = 7.0 Hz, 1H), 4.67 (s, 1H), 4.47 (s, 1H), 4.00 (t, J = 6.5 Hz, 2H), 3.65 (s, 3H), 1.94 (s, 3H), 1.85 (q, J = 7.0 Hz, 2H), 1.55 (s, 3H), 1.46 (s, 3H), 1.06 (t, J = 7.4 Hz, 3H); ^{13}C NMR (151 MHz, Chloroform-d) δ 172.85 (C=O), 170.94 (C=O), 170.66 (C=O), 166.49 (C=O), 164.59 (C=O), 163.87, 160.08, 156.66, 132.21, 128.38, 128.13, 120.32, 116.35, 114.39, 85.95, 79.85 (OCH_2O), 70.12, 69.81, 68.00, 64.88, 61.21, 60.41, 58.80, 50.46, 31.57, 26.71, 22.41, 19.78, 10.42.

HR-MS (ESI) Calcd for $C_{32}H_{37}N_3O_{10}S$ ($M + H^+$): 656.7790; Found: 656.7194; purity: 98.75% (determined by HPLC).

2.4.4 | ([4-Nitrobenzoyl]oxy)methyl (*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (5d)

Light-yellow solid; yield: 71.24%; Rf: 0.47; IR (KBr, cm^{-1}): 3,369.64 (OH), 2,974.23, 2,374.37, 1,780.30 (C=O), 1,670.35, 1,600.92, 1,521.84, 1,444.68, 1,269.16, 1,166.93, 1,087.85; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.07 (d, *J* = 6.8 Hz, 1H, CONH), 8.29 (dd, *J* = 51.0, 8.0 Hz, 5H), 7.18 (d, *J* = 7.6 Hz, 2H), 6.91 (d, *J* = 8.8 Hz, 1H, NH), 6.73 (d, *J* = 7.6 Hz, 2H), 6.12–6.07 (m, 2H, OCH_2O), 5.65 (dd, *J* = 8.8, 4.1 Hz, 1H), 5.55 (d, *J* = 4.0 Hz, 1H), 5.03 (d, *J* = 7.0 Hz, 1H), 4.68 (s, 1H), 4.50 (s, 1H), 3.65 (s, 3H), 1.94 (s, 3H), 1.57 (s, 3H), 1.47 (s, 3H); ^{13}C NMR (151 MHz, Chloroform-d) δ 172.85 (C=O), 170.85 (C=O), 170.72 (C=O), 166.30 (C=O), 163.12 (C=O), 160.09, 156.68, 151.14, 133.70, 131.19, 128.35, 128.04, 123.80, 116.36, 116.00, 86.01, 80.32 (OCH_2O), 70.08, 68.06, 64.79, 61.20, 60.41, 58.88, 52.36, 50.48, 49.84, 31.61, 30.13, 19.78.

HR-MS (ESI) Calcd for $C_{29}H_{30}N_4O_{11}S$ ($M + H^+$): 643.6360; Found: 643.6357; purity: 98.86% (determined by HPLC).

2.4.5 | ((4-(*N*,*N*-Dipropylsulfamoyl)benzoyl)oxy)methyl (*Z*)-6-(2-(4-hydroxyphenyl)-2-([4-methoxy-4-oxobut-2-en-2-yl]amino)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (5e)

Light-yellow solid; yield: 76.76%; Rf: 0.59; IR (KBr, cm^{-1}): 3,365.78 (OH), 2,968.45, 2,374.37, 1,782.23 (C=O), 1,664.57, 1,602.85, 1,510.26, 1,450.47, 1,336.67, 1,269.16, 1,163.08, 1,087.85; ^{1}H NMR (600 MHz, Chloroform-d) δ 9.07 (d, J = 7.0 Hz, 1H, CONH), 8.18 (d, J = 8.1 Hz, 2H), 7.91 (d, J = 8.2 Hz, 2H), 7.17 (d, J = 8.1 Hz, 2H), 6.99 (d, J = 8.8 Hz, 1H, NH), 6.75–6.67 (m, 2H), 6.07 (dd, J = 24.9, 5.7 Hz, 2H,OCH_2O), 5.63 (dd, J = 8.8, 4.1 Hz, 1H), 5.55 (d, J = 4.0 Hz, 1H), 5.03 (d, J = 7.0 Hz, 1H), 4.66 (s, 1H), 4.50 (d, J = 13.6 Hz, 1H), 3.64 (s, 3H), 3.12 (t, J = 7.7 Hz, 4H), 1.93 (s, 3H), 1.61–1.52 (m, 7H), 1.46 (s, 3H), 0.88 (t, J = 7.3 Hz, 6H); ^{13}C NMR (151 MHz, DMSO-d_6) δ172.82 (C=O), 171.03 (C=O), 170.83 (C=O), 166.35 (C=O), 163.65 (C=O), 160.17, 156.84, 145.35, 131.62, 130.68, 128.34, 127.94, 127.22, 116.36, 85.88, 80.23 (OCH_2O), 70.08, 68.02, 64.80, 61.17, 58.90, 50.50, 49.95, 31.60, 26.72, 21.94, 19.80, 11.14.

HR-MS (ESI) Calcd for $C_{35}H_{44}N_4O_{11}S_2$ ($M + H^+$): 761.8740; Found: 761.8734; purity: 97.31% (determined by HPLC).

2.4.6 | (Benzoyloxy)methyl 6-(2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (6a)

Light-yellow solid; yield: 87.48%; solubility: 574.06 mg/ml; Rf: 0.73; IR (KBr, cm^{-1}): 3,409.5 (NH_2), 3,201.3 (OH), 2,975.6, 2,613.1, 1,770.3 (C=O), 1,689.3, 1,606.4, 1,515.8, 1,459.8, 1,268.9, 1,089.6; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.83 (s, 1H, OH), 9.28 (d, *J* = 7.5 Hz, 1H, CONH), 8.69 (s, 2H, NH_2), 7.96 (d, *J* = 7.7 Hz, 2H), 7.71 (t, *J* = 7.4 Hz, 1H), 7.56 (t, *J* = 7.6 Hz, 3H), 7.28 (d, *J* = 8.1 Hz, 2H), 6.79 (d, J = 8.2 Hz, 2H), 6.03 (dd, J = 47.9, 6.0 Hz, 2H, OCH_2O), 5.58 (dd, J = 7.0, 3.9 Hz, 1H), 5.45 (d, J = 4.1 Hz, 1H), 4.95 (s, 1H), 4.42 (s, 1H), 1.47 (s, 3H), 1.31 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.33 (C=O), 168.51 (C=O), 166.81 (C=O), 164.78 (C=O), 158.65, 134.74, 129.93, 129.57, 128.60, 124.18, 115.77, 80.83 (OCH_2O), 70.10, 67.39, 64.38, 58.67, 54.98, 29.99, 26.66.

HR-MS (ESI) Calcd for $C_{24}H_{25}N_3O_7S$ ($M + H^+$): 499.5380; Found: 499.5367; purity: 97.11% (determined by HPLC).

2.4.7 | ([4-Methoxybenzoyl]oxy)methyl 6-(2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (6b)

Light-yellow solid; yield: 86.34%; solubility: 142.19 mg/ml; Rf: 0.72; IR (KBr, cm^{-1}): 3,459.7 (NH_2), 3,214.8 (OH), 2,973.7, 2,591.9, 1,772.3 (C=O), 1,722.1, 1,666.2, 1,606.4, 1,515.8, 1,457.9, 1,263.1, 1,168.7, 1,095.4; ^{1}H NMR (600 MHz, DMSO-d_6) δ9.77 (s, 1H, OH), 9.27 (d,

J = 7.6 Hz, 1H, CONH), 8.64 (s, 2H, NH_2), 7.92 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 7.08 (d, J = 8.1 Hz, 2H), 6.78 (d, J = 7.8 Hz, 2H), 6.00 (dd, J = 51.1, 6.0 Hz, 2H,OCH_2O), 5.59 (dd, J = 7.8, 4.1 Hz, 1H), 5.45 (d, J = 4.0 Hz, 1H), 4.94 (s, 1H), 4.41 (s, 1H), 3.84 (s, 3H), 1.47 (s, 3H), 1.30 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.35 (C=O), 168.41 (C=O), 166.83 (C=O), 164.48 (C=O), 164.36, 158.65, 132.20, 129.64, 124.05, 120.63, 115.80, 114.86, 80.60 (OCH_2O), 70.12, 67.33, 64.39, 58.60, 56.12, 55.02, 29.87, 26.66.

HR-MS (ESI) Calcd for $C_{25}H_{27}N_3O_8S$ (M + H^+): 530.5640; Found: 530.5637; purity: 98.34% (determined by HPLC).

2.4.8 | ([4-Ethoxybenzoyl]oxy)methyl 6-(2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (6c)

Light-yellow solid; yield: 88.47%; solubility: 220.38 mg/ml; Rf: 0.77; IR (KBr, cm^{-1}): 3,398.1 (NH_2), 3,205.1 (OH), 2,967.9, 2,599.6, 1772.3 (C=O), 1,693.2, 1,604.5, 1,513.8, 1,467.6, 1,261.2, 1,168.7, 1,087.7; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.78 (s, 1H, OH), 9.25 (d, J = 7.5 Hz, 1H, CONH), 8.56 (s, 2H, NH_2), 7.90 (d, J = 8.3 Hz, 2H), 7.27 (d, J = 8.1 Hz, 2H), 7.06 (d, J = 8.3 Hz, 2H), 6.78 (d, J = 8.0 Hz, 2H), 5.99 (dd, J = 50.3, 6.1 Hz, 2H, OCH_2O), 5.59 (d, J = 4.9 Hz, 1H), 5.45 (d, J = 3.9 Hz, 1H), 4.92 (s, 1H), 4.41 (d, J = 1.5 Hz, 2H), 4.02 (t, J = 6.9 Hz, 2H), 1.47 (s, 3H), 1.30 (s, 3H), 0.97 (t, J = 7.4 Hz, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.39 (C=O), 168.62 (C=O), 166.84 (C=O), 164.36 (C=O), 163.83, 158.60, 132.20, 129.58, 124.37, 120.44, 115.76, 115.24, 80.58 (OCH_2O), 70.02 (d, J = 29.2 Hz), 67.37, 64.38, 60.20, 58.61, 55.09, 29.92, 26.66, 22.31, 10.73.

HR-MS (ESI) Calcd for $C_{27}H_{31}N_3O_8S$ (M + H^+): 558.6180; Found: 558.6174; purity: 96.83% (determined by HPLC).

2.4.9 | ([4-Nitrobenzoyl]oxy)methyl 6-(2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (6d)

Light-yellow solid; yield: 89.67%; solubility: 484.84 mg/m; Rf: 0.72; IR (KBr, cm^{-1}): 3,428.8 (NH_2), 3,224.4 (OH), 2,975.6, 2,611.1, 1,766.5 (C=O), 1,691.3, 1,610.3, 1,525.4, 1,353.8, 1,270.9, 1,089.6; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.78 (s, 1H, OH), 9.26 (d, *J* = 7.6 Hz, 1H, CONH), 8.59 (s, 2H, NH_2), 8.37 (d, *J* = 8.1 Hz, 2H), 8.20 (d, *J* = 8.0 Hz, 2H), 7.27 (d, *J* = 7.9 Hz, 2H), 6.78 (d, *J* = 7.9 Hz, 2H), 6.07 (dd, *J* = 38.6, 6.2 Hz, 2H, OCH_2O), 5.59 (t, *J* = 5.6 Hz, 1H), 5.46 (d, *J* = 4.1 Hz, 1H), 4.94 (s, 1H), 4.45 (s, 1H), 1.48 (s, 3H), 1.31 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.29 (C=O), 168.52 (C=O), 166.76 (C=O), 163.45 (C=O), 158.62, 151.21, 134.00, 131.47, 129.62, 124.61, 124.21, 115.77, 81.24 (OCH_2O), 70.06, 67.40, 64.41, 58.70, 55.04, 30.06, 26.70.

HR-MS (ESI) Calcd for $C_{24}H_{24}N_4O_9S$ (M + H^+): 545.5350; Found: 545.5356; purity: 98.34% (determined by HPLC).

2.4.10 | ((4-(*N*,*N*-Dipropylsulfamoyl)benzoyl)oxy) methyl 6-(2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (6e)

Light-yellow solid; yield: 87.25%; solubility: 442.48 mg/ml; Rf: 0.75; IR (KBr, cm^{-1}): 3,405.7 (NH_2), 3,209.1 (OH), 2,967.9, 299.6, 1,762.6 (C=O), 1,687.4, 1,606.4, 1,515.8, 1,463.7, 1,334.5, 1,270.9, 1,159; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.79 (s, H, OH) 9.24 (s, 1H, CONH), 8.30 (s, 2H, NH_2), 8.14 (d, J = 8.1 Hz, 2H), 7.97 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 8.2 Hz, 2H), 6.78 (d, J = 8.2 Hz, 2H, OCH_2O), 6.08 (dd, J = 39.4, 6.5 Hz, 2H, OCH_2O), 5.58 (d, J = 4.0 Hz, 1H), 5.46 (d, J = 4.1 Hz, 1H), 4.91 (s, 1H), 4.45 (s, 1H), 3.05 (dd, J = 8.5, 6.5 Hz, 4H), 1.50–1.41 (m, 7H), 1.32 (s, 2H), 0.79 (t, J = 7.3 Hz, 6H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.41 (C=O), 169.05 (C=O), 166.80 (C=O), 163.80 (C=O), 158.50, 144.85, 131.99, 130.98, 129.47, 127.86, 124.98, 115.71, 81.13 (OCH_2O), 70.05, 67.47, 64.40, 58.70, 55.25, 50.00, 30.14, 26.68, 21.99, 11.39.

HR-MS (ESI) Calcd for $C_{30}H_{38}N_4O_9S_2$ (M + H^+): 663.7730; Found: 663.7737; purity: 97.24% (determined by HPLC).

2.5 | Pharmacology

The MIC of hybrid molecules was determined by dilution in a liquid medium distributed in a 96-well plate according to the standard protocol set by the Clinical and Laboratory Standards Institute. The results of antibacterial testing of hybrid molecules (**6a–e**) against a panel of selected bacteria (*S. aureus* American Type Culture Collection [ATCC] 29,213, ATCC 11632; MRSA 11; *E. coli* ATCC 25922; *Salmonella* LS677, GD836, GD828, GD3625) are reported in Table 1. These plates were incubated for 24 hr at 37 °C. The lowest concentration which inhibited the growth of the test organism was considered to be the MIC (μg/ml). Amoxicillin was used as the control drug.

2.6 | Systemic infections in mice

Female ICR mice weighing about 18–22 g (Changsha, China) were infected intraperitoneally with 0.5 ml of bacterial suspension. The strains and the challenge dose per mouse were as follows: *S. enteritidis* GD836, 1.35×10^9 cfu. *Salmonella enteritidis* GD836 were injected as suspensions in normal saline. **6a–e** (hydrochloride) and amoxicillin (Sodium salt) were orally administered in 0.2 ml of aqueous solution at 0.5 and 4 hr after pathogen inoculation. The number of mice that survived in each experimental group was monitored up to 48 hr after pathogen inoculation, and the 50% effective dose (ED_{50}) of the drug-treated animals were determined by the probit method. Each experimental group consisted of 10 animals and four different doses of the drug was evaluated per compound, and physiological saline was used as the vehicle control. (Bliss, 1934).

TABLE 1 In vitro antimicrobial activities of compound **6a–e** based on MIC values

	MIC (μg/ml)							
	Gram-Positive				Gram-Negative			
Compound	*S. aureus* ATCC 29213	*S. aureus* ATCC 11632	*S. aureus* MRSA 11	*E. coli* ATCC 25922	*S. enteritidis* LS677	GD836	GD828	GD3625
6a	4	8	>128	16	2	4	4	8
6b	4	8	128	8	2	4	4	4
6c	4	16	128	16	4	8	8	2
6d	4	8	64	8	2	2	2	2
6e	4	8	128	8	2	4	4	4
Amoxicillin	2	4	>128	4	2	8	8	2

3 | RESULTS AND DISCUSSION

3.1 | Chemistry

Our research focuses on the design, synthesis, and bioactivity evaluation of a series of hybrid molecules between amoxicillin and derivatives of benzoic acid. The excellent antibacterial activity of sultamicillin proves that hybrid molecules connected by methylene bridges have great development potential (Friedel, Campoli-Richards, & Goa, 1989). In addition, a series of studies have shown that benzoic acid derivatives play a positive role in enhancing the antibacterial properties of compounds.(Begini et al., 2020; Filho, Kronenberger, Ferreira, Trossini,& Marcio, 2020; Sharma, Parvinder, Kakkar, & Khatkar, 2020), and the latest research shows that benzoic acid derivatives with para-substituents have has potential as antibiofilm agent (Campbell et al., 2020; Zarafu, 2020). These studies led us to design and synthesise a series of hybrid molecules which were linked via a methylene bridge in order to investigate the effect of the presence of benzoic acid derivatives on the biological activity.

The synthesis of new compounds is shown in Scheme 1. Compound **2a–e** was converted to the corresponding iodomethyl derivative by the Finkelstein reaction to give compound **4a–e**. Then, compound **4a–e** was condensed with the carboxylate ion of compound **2** at 0–5 °C to give compound **5a–e**. The condensation product was dissolved in an aqueous acetone solution. The pH of this solution was adjusted to 2.5 with 10% HCl, the protecting group was removed, and then the acetone in the solution was removed by vacuum spinning. Then, excess NaCl was added to the remaining solution to precipitate the product. Finally, the crude product was purified by column chromatography using silica gel to afford compound **6a–e**.

3.2 | In vitro antibacterial activity

The antimicrobial activity of hybrids (**6a–e**) in vitro was evaluated. Their MIC values against Gram-positive and Gram-negative bacteria were determined by comparison with that of amoxicillin (Table 1).

TABLE 2 Efficacy in systemic infections in mice

	ED_{50} (mg/kg)
Compound	*S. enteritidis* GD836
6a	14.6575
6b	13.2496
6c	16.6281
6d	14.3089
6e	16.9435
Amoxicillin	17.5997

The MIC values of the hybrid molecules of the five newly synthesized amoxicillin–benzoic acid analogs indicated that certain hybrid molecules had comparable or better activity against *Salmonella* species than amoxicillin. Compound **6d** exhibited the most potent inhibitory activity against *Salmonella enteritidis* GD828 (MIC = 2 μg/ml), and *Salmonella enteritidis* GD836 (MIC = 2 μg/ml), which were fourfold more potent than that of amoxicillin, respectively. Conversely, the in vitro activity of most of the test compounds against *E. coli* species (Gram-positive bacteria) was comparable with (or even weaker) than that of amoxicillin. However, compound **6d** was superior to that of amoxicillin against MRSA 11 (MIC = 64 μg/ml).

3.3 | In vivo antibacterial activity

The protective effect of **6a–e** against systemic infections with Salmonella enteritidis GD836 in mice were compared with Amoxicillin (Table 2).

Compound **6b** showed the greatest efficacy of all the compounds tested against systemic infections caused by Salmonella enteritidis GD836. Although **6a–e** has good in vitro activity against Salmonella enteritidis GD836, their in vivo antibacterial activity is not significantly superior to amoxicillin. This unexpected loss of in vivo activity of these derivatives may partly be due to the poor bioavailability of

these hybrid molecules when administered orally, because they may have poor stability in the digestive tract.

4 | CONCLUSIONS

Five hybrid molecules similar to amoxicillin–benzoic acid were synthesized and evaluated. Amoxicillin–benzoic acid-like hybrid molecules based on a diester structure could improve the activity of amoxicillin against *Salmonella* species. Compound **6d** had good activity against MARSA 11. Our findings provided a new approach to researching and developing a novel antibiotic.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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