

Synthesis of typical sulfonamide antibiotics with [¹⁴C]- and [¹³C]-labelling on phenyl ring for environmental studies

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Abstract

Background

As a kind of widely used antibiotics, sulfonamide antibiotics (SAs) has become ubiquitous environmental contaminants that caused public concerns. The behavior of SAs in complex environmental system need to be elucidated, which is hampered by unavailability or high cost of isotope-labelled SAs.

Results

Using commercially available uniformly [^{14}C]- and [^{13}C]-labelled aniline as starting material, we synthesized [phenyl-ring- ^{14}C]- and [phenyl-ring- ^{13}C]-labelled sulfamethoxazole (SMX), sulfamonomethoxine (SMM), and sulfadiazine (SDZ) using four-step (via condensation of labelled *N*-acetylsulfanilyl chloride and aminoheterocycles) or five-step (via condensation of labelled *N*-acetylsulfonamide and chloroheterocycles) reactions in good yields (5.0–22.5% and 28.1–54.1% for [^{14}C]- and [^{13}C]-labelled SAs, respectively) and high purities (> 98.0%).

Conclusion

The synthesis of [^{14}C]-labelled SAs could be completed on milligram-level, being feasible for preparation of labelled SAs with high specific radioactivity. This study provides efficient and maneuverable methods to obtain a variety of [^{14}C]- or [^{13}C]-labelled SAs for studies on their environmental behavior, such as fate, transformation, and bioaccumulation.

Background

Sulfonamide antibiotics (SAs) are widely used in the treatment of human disease and in modern livestock. Due to their low biodegradation and insufficient removal in waste water treatment plant [1, 2], high concentrations of sulfonamides, such as sulfadiazine (SDZ), sulfamethoxazole (SMX), and sulfamonomethoxine (SMM), are widely detected in environmental media such as agroecosystem, sediments, and rivers [3–5]. After entering the environment, SAs exert adverse effects on organisms [6–9], and the environment hazards and risk of SAs have caused a widely concern. A comprehensive understanding of SAs in environment, including its adsorption, biodegradation, transformation, formation of non-extractable residues (NERs), and transport, helps assess their environment risks.

Techniques using [^{14}C]-radioactive and [^{13}C]-stable isotopes are often used to study the environmental behavior of pollutants. For example, [^{14}C]-tracer with low detection limit and convenient handling with complex environmental samples is used to investigate the environmental fate especially the mineralization and NERs of organic pollutants such as pesticides, brominated flame retardants, alkylphenols, and polycyclic aromatic hydrocarbons [10–14]. Using mass spectrometry and nuclear

magnetic resonance (NMR) spectroscopy as analytical tools, stable isotopes (e.g., ^{13}C , ^{15}N) helps quantify and identify metabolites of the pollutants in complex matrices [15–20]. Phospholipid fatty acid analysis and DNA stable-isotope probing using [^{13}C]-tracers are powerful tools for analysis of microbial biomass and community composition [21, 22]. Therefore, [^{14}C]- and [^{13}C]-labelled SAs are increasing in need, however are either commercially unavailable or commercially too expensive. Efficient and easy-operating “in house” syntheses of [^{14}C]- and [^{13}C]-SAs, especially on micro-scales with good yields, are highly desired.

Compared with conventional synthetic method, a successful synthesis of [^{14}C]-SAs on a micro-scale requires stable solvents, suitable reaction conditions, and simple purification methods of each product [23]. The conventional synthetic method of unlabelled SAs was a four-step route, including acetylation of aniline using acetic anhydride, chlorosulfonation of *N*-acetylaniline with ClSO_3H , condensation of sulfonyl chlorides with nucleophiles such as amines, and alkaline hydrolysis of the acetyl-protecting group [24–27]. It is however notable that the synthetical conditions in the route described above was suitable for synthesis of SAs at gram-level and cannot be applied to synthesis of SAs on a micro-scale (milligram-level) due to the difficulty of mixing in solvent-free condition and crystalizing of products. In a previous study, [^{14}C]-SDZ labelled on the heterocyclic ring was prepared by reaction of *N*-acetylsulfanilyl chloride with [^{14}C]-2-aminopyrimidine [28], while SDZ and other common types of SAs (such as SMM and SMX) labelled with carbon-14 on the phenyl ring, which are required to trace the transformation of phenyl ring of SAs, has not been described yet.

In this study, we reported synthetical methods for preparation of typical SAs with [^{14}C]- or [^{13}C]-labelling on the phenyl ring in good yields, especially the synthesis of [^{14}C]-labelled SAs on a micro-scale (milligram-level), which can be employed to prepare a variety of [^{14}C]- or [^{13}C]-labelled SAs.

Materials And Methods

Chemicals

Uniformly [phenyl-ring- ^{14}C]-labelled aniline hydrochloride (**1a**, Fig. 1, 2.96×10^9 Bq/mmol, 99% radiochemical purity) and Uniformly [phenyl-ring-U- ^{13}C]-labelled aniline hydrochloride (**1b**, Fig. 1, 99% of ^{13}C atom, 98% chemical purity) were purchased from Moravek Inc (California, USA) and Alsachim (Illkirch Graffenstaden, France), respectively. Unlabelled SDZ, SMM, SMX, *N*-acetylaniline, and *N*-acetylsulfanilyl chloride (purity $\geq 99\%$), were purchased from J&K Co. (Shanghai, China). All other reagents were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China) and were of analytical purity grade. Pyridine was dried over 4 Å molecular sieves for at least 48 h prior to use.

Analyses

The reaction products were purified by flash column chromatography (CHEETAH TMMP100; Agela, Tianjin, China) or preparative thin-layer chromatography (TLC, GF254, 1 mm, 20 × 20 cm; Huanghai, Shandong, China). The purity of products was analyzed by analytical thin-layer chromatography (TLC, GF254, 0.25 mm, 3 cm × 10 cm, Huanghai, Shandong, China) coupled with an imaging scanner (Typhoon Trio⁺; GE Healthcare, U.S.), or high-performance liquid chromatography (HPLC, 1100 system; Agilent Technology, USA). HPLC (1260 system; Agilent Technology, USA) coupled to a Q-TOF tandem mass spectrometer (HPLC-Q-TOF-MS/MS, triple TOF 5600 system; AB SCIEX, USA), and NMR spectroscopy (AVANCE III HD-500; Bruker, Germany) were used to identify synthesized products. Radioactivity was determined by liquid scintillation counting (LSC, LS6500; Beckman Counter, USA). Details of the instruments used in the purifications and analyses are provided in the Supporting Information (**SI**).

Syntheses

Synthesis of [¹⁴C]-SMX (5a), [¹⁴C]-SMM (7a)

Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylaniline (2a)

To ¹⁴C-labelled aniline hydrochloride (**1a**, 3.70×10^8 Bq, 1.11×10^9 Bq/mmol, 99.0% purity) in deionized water (10 mL) were K₂CO₃ powder (360 mg) and acetic anhydride (187 µL) sequentially added with stirring at 25°C (Fig. 1). The mixture was stirred at 25°C for 1 h and then extracted five times with ethyl acetate (10 mL each). The extract was dried with anhydrous Na₂SO₄ and evaporated under vacuum to ~1 mL. The product in the extract was purified by flash chromatography (For details, see **SI.1**) with an elution gradient (**SI, Table S1**), giving **2a** (3.33×10^8 Bq, 1.11×10^9 Bq/mmol) in 90.0% yield. TLC analysis using petroleum ether: ethyl acetate (1:4 / v: v), containing 0.2% CH₃COOH as eluent (*R_f* value of **2a** = 0.45) coupled to autoradiography (**SI.2**) showed a radiochemical purity of 99.0%.

Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylsulfanilyl chloride (3a)

To **2a** (2.59×10^8 Bq, 2.96×10^9 Bq/mmol, 99.0% purity) in CCl₄ (1 mL) was ClSO₃H (45 µL) added dropwise with stirring in an ice bath. The mixture was then stirred at 58°C for 2 h, NaCl (4 mg) was added (Fig. 1, Method I). The reaction mixture was stirred for another 2 h and then cooled down to the room temperature. The residual chlorosulfonic acid in the mixture was hydrolyzed with ice-cold water (10 mL), and the mixture was extracted twice with ethyl acetate (35 mL each). The extracts were dried with anhydrous Na₂SO₄ and evaporated to ~1 mL. The product in the extract was purified by flash chromatography (**SI.1**) with an elution gradient (**SI, Table S1**), giving **3a** (1.39×10^8 Bq, 2.96×10^9 Bq/mmol) in 53.9% yield. TLC analysis using petroleum ether: ethyl acetate (1:4 / v: v), containing 0.2% CH₃COOH as eluent (*R_f* value of **3a** = 0.35) coupled to autoradiography (**SI.2**) showed a radiochemical purity of 96.0%.

Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylsulfamethoxazole (4a), Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylsulfamonomethoxine (6a)

Synthesis of **4a:** To **3a** (4.07×10^5 Bq, 7.40×10^6 Bq/mmol) in 200 uL acetone were 3-amino-5-methylisoxazole (**11**, 13 mg), anhydrous pyridine (11 μ L), and 5 pieces of molecular sieves (4 Å with a diameter of 1 mm) added with stirring in an ice bath, and then stirred for 5 h at 60°C. The resulting mixture were then diluted with methanol (200 μ L) and separated by preparative TLC using petroleum ether: ethyl acetate (1:4 / v: v) containing 0.2% CH₃COOH as eluent. The product band of **4a** ($R_f = 0.53$) was scraped from the TLC plate and extracted six times with ethyl acetate (15 mL each). After concentration by evaporation, **4a** was obtained (2.07×10^5 Bq, 7.40×10^6 Bq/mmol, 51.0% yield) with 95.0% purity as determined by TLC coupled to autoradiography (**SI.2**).

Synthesis of **6a:** To **3a** (3.7×10^7 Bq, 7.55×10^8 Bq/mmol) in 200 uL acetone were 4-amino-6-methoxypyrimidine (**12**, 16.1 mg), anhydrous pyridine (10 μ L) and 5 pieces of molecular sieves (4 Å with a diameter of 1 mm) sequentially added with stirring in an ice bath, and then stirred for 23 h at 60°C. The subsequent purification of **6a** ($R_f = 0.18$) was the same as for **4a**. **6a** (5.77×10^6 Bq, 7.55×10^8 Bq/mmol, 15.6% yield) was obtained with 95.0% purity as determined by TLC coupled to autoradiography (**SI.2**).

Uniformly [phenyl-ring-¹⁴C]-SMX (5a), Uniformly [phenyl-ring-¹⁴C]-SMM (7a)

4a (2.04×10^5 Bq, 7.40×10^6 Bq/mmol), **6a** (4.81×10^6 Bq, 7.55×10^8 Bq/mmol) were heated in NaOH solution (10%, 1 mL) for 3 h at 100°C and neutralized with 6 M HCl to pH 6. The products were extracted with ethyl acetate (15 mL each) eight times. The extracts were dried with anhydrous Na₂SO₄, evaporated to around 0.5 mL, and purified by preparative TLC using petroleum ether: ethyl acetate (1:4 / v: v) containing 0.4% CH₃COOH as eluent. The product bands of **5a** and **7a** ($R_f = 0.6$ and 0.51, respectively) were scraped from the plates and extracted with ethyl acetate (15 mL each) six times. The extracts were evaporated to around 0.1 mL, giving **5a** (1.85×10^5 Bq) and **7a** (3.18×10^6 Bq) in 90.9% and 66.2% yield, respectively, with purities of 98.1% and 98.3%, respectively, as determined by HPLC (**SI.4**). Chemical structure of **5a** and **7a** were characterized by ¹H-NMR, ¹³C-NMR (**SI.5**) and LC-Q-TOF-MS/MS (**SI.6**) using the corresponding unlabelled compounds synthesized with the same procedures.

Synthesis of [¹⁴C]-SDZ (10a)

Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylsulfanilyl chloride (3a)

To **2a** (2.48×10^8 Bq, 1.11×10^9 Bq/mmol, 99.0% purity) in CCl₄ (0.5 mL) was ClSO₃H (170 μ L) added dropwise with stirring in an ice bath. The mixture was stirred at 58°C for 2 h, followed by addition of SOCl₂ (25 μ L) and another 2 h of heating at 58°C (Fig. 1, Method II). After the reaction, the mixture was cooled down to room temperature and extracted twice with ethyl acetate (35 mL each). The extracts were dried with anhydrous Na₂SO₄ and evaporated to ~1 mL. The purity of **3a** (1.98×10^8 Bq) in the mixture was 93.0% as determined by TLC using petroleum ether: ethyl acetate (1:4 / v: v), containing 0.2% CH₃COOH as eluent (R_f value of **3a** = 0.35) coupled to autoradiography. The mixture without purification was directly used for subsequent synthesis of **8a**. The yield of **3a** according to its purity in the mixture was 74.3%.

Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylsulfonamide (8a)

The mixture containing crude **3a** (1.98×10^8 Bq, 6.29×10^8 Bq/mmol, 93.0% purity) was mixed with acetone (1 mL), after which ammonium hydroxide (0.5 mL, 28% NH₃ in water) was added dropwise at 0°C. The mixture was vigorously stirred at room temperature for 1 h and the pH was adjusted to 6 with 6 M HCl. It was then extracted eight times with ethyl acetate (15 mL each), dried with anhydrous Na₂SO₄. The extract was evaporated and the product was purified by flash chromatography with an elution gradient (**Table S1**), resulting in **8a** (1.81×10^8 Bq) in 98.3% yield with a purity of 99.0% as analyzed by TLC using petroleum ether: ethyl acetate (1:4 / v: v), containing 0.2% CH₃COOH as eluent (*R_f* value of **8a** = 0.26) coupled to autoradiography.

Uniformly [phenyl-ring-¹⁴C]-labelled N-acetylsulfadiazine (9a)

To **8a** (1.74×10^8 Bq, 6.29×10^8 Bq/mmol, 99.0% radiochemical purity) in *N,N*-dimethylacetamide (800 μL) were 2-chloropyrimidine (**13**, 48.7 mg) and K₂CO₃ (58.6 mg) added sequentially with stirring at room temperature. The mixture was heated at 150°C for 4.5 h, and the solvent *N,N*-dimethylacetamide was then removed by evaporation. The crude product was dissolved in water and cooled in an ice bath. The mixture was adjusted to pH 6 with 6 M HCl and the precipitates were washed with ice-cold water, resulting in **9a** (1.10×10^8 Bq) with 57% purity as analyzed by TLC (**SI.2**) coupled to autoradiography (*R_f* = 0.13) using petroleum ether: ethyl acetate (1:4 / v: v), containing 0.2% CH₃COOH as eluent. The yield of **9a** according to its purity was 36.0%.

Uniformly [phenyl-ring-¹⁴C]-labelled SDZ (10a)

The crude **9a** (9.25×10^7 Bq, 6.29×10^8 Bq/mmol, 57.0% radiochemical purity) was reacted with NaOH solution (10%, 5 mL) for 3 h at 100°C and neutralized with 6 M HCl to pH 6. The products were extracted with ethyl acetate (15 mL each) eight times. The extracts were dried with anhydrous Na₂SO₄, evaporated to around 0.5 mL. The crude product was then recrystallized from boiling methanol. The precipitates were centrifuged and washed three times with methanol, resulting in **10a** (3.11×10^7 Bq, 6.29×10^8 Bq/mmol). The purity of **10a** was 98.3% as determined by HPLC (*t_R* = 5.73 min. For details, see **SI**). The supernatant was further extracted five times with ethyl acetate (15 mL each), which was dried with anhydrous Na₂SO₄ and evaporated to dryness, giving solids containing **10a**. The solid product was mixed with unlabelled SDZ (54 mg) and then recrystallized from boiling methanol. The precipitate was washed three times with methanol, resulting in another portion of **10a** with a low specific activity (1.10×10^7 Bq, 7.40×10^7 Bq/mmol) with a radiochemical purity of 98.3%. The total amount of **10a** was 4.21×10^7 Bq with a total yield of 79.9%. The chemical structure of **10a** were characterized by ¹H-NMR, ¹³C-NMR (**SI.5**) and LC-Q-TOF-MS/MS (**SI.6**) using the corresponding unlabelled compounds synthesized with the same procedures.

Synthesis of [¹³C]-SMX (5b), [¹³C]-SMM (7b), and [¹³C]-SDZ (10b)

Uniformly [phenyl-ring-¹³C]-labelled N-acetylaniline (2b)

To ¹³C-labelled aniline hydrochloride (**1b**, 3.00 g, 99% of ¹³C atom) in a 200-mL flask were K₂CO₃ solution (0.32 g/mL, 30 mL) and acetic anhydride (4.70 g) added sequentially with stirring at 25°C. The mixture was further stirred at 25°C for 1 h and then extracted five times with ethyl acetate (15 mL each). The extract was washed with 20 mL of H₂O and then evaporated, resulting in **2b** (3.01 g, 99% of ¹³C atom, 99.0% purity (For detail, see **SI.4**) in 95.7% yield.

Uniformly [phenyl-ring-¹³C]-labelled N-acetylsulfanilyl chloride (3b)

To [¹³C]-**2** (3.00 g, 99% of ¹³C atom) in CCl₄ (5 mL) was ClSO₃H (19.8 g) added dropwise with stirring in an ice bath. The mixture was stirred at 58°C for 2 h and SOCl₂ (2.67 g) was then added (Fig. 1, Method II). The mixture was heated at 58°C for another 2 h and cooled down to room temperature. White crystals were formed after dropwise addition of ice-cold water (10 mL) to the mixture and were washed twice with ice-cold water (each 10 mL) by filtration, resulting in **3b** (4.29 g, 99% of ¹³C atom, 96.0% purity (**SI.4**) in 82.8% yield.

Uniformly [phenyl-ring-¹³C]-labelled N-acetylsulfamethoxazole (4b), N-acetylsulfamonomethoxine (6b), and N-acetylsulfadiazine (9b)

Synthesis of **4b**: To **3b** (500 mg, 99% of ¹³C atom) in acetone (2 mL) were 3-amino-5-methylisoxazole (**11**, 412 mg), anhydrous pyridine (339 μL) and 10 pieces of molecular sieves (diameter 1 mm, 4 Å) were added sequentially with stirring in an ice bath. The mixture was then stirred for 7 h at 60°C. The molecular sieves were removed and acetone was evaporated. The crude product **4b** (470 mg, 99% of ¹³C atom, 95.0% purity (**SI.4**) was obtained in 73.8% yield after crystallization in ice-cold water.

Synthesis of **6b**: To **3b** (500 mg, 99% of ¹³C atom) in acetone (2 mL) were 4-amino-6-methoxypyrimidine (**12**, 526 mg), anhydrous pyridine (339 μL, 4.2 mmol), and 10 pieces of molecular sieves (diameter 1 mm, 4 Å) added with stirring in an ice bath. The reaction conditions and workup were similar to the synthesis of **4b** as describe above. The crude product **6b** (286 mg, 99% of ¹³C atom, 93.0% purity (**SI.4**) was obtained in 42.3%.

Synthesis of **8b**: To **3b** (1.0 g, 99% of ¹³C atom) in acetone (10 mL) were ammonium hydroxide (5 mL, 28% NH₃ in water) added with stirring in an ice bath. The mixture was vigorously stirred at 25°C for 1 h. Then acetone was removed by evaporation. After addition of ice-cold water and adjusting with 6 M HCl to pH about 6, **8b** (672 mg, 99% of ¹³C atom, 98.0% purity (**SI.4**) was obtained in 73.0% by filtration and washing with ice-cold water.

Synthesis of **9b**: To **8b** (450 mg, 99% of ¹³C atom) in *N,N*-dimethylacetamide (3.5 mL) were 2-chloropyrimidine (**13**, 361 mg), K₂CO₃ (439 mg) added with stirring in room temperature. Then the mixture

was stirred for 5 h at 150°C. The subsequent procedures were similar to the synthesis of **9a** as described above, to obtain **9b** (494 mg, 99% of ¹³C atom, 93.0% purity (**SI.4**)) in 74.8% yield.

Uniformly [phenyl-ring-¹³C]-labelled SMX (5b), SMM (7b), and SDZ (10b)

4b (300 mg, 99% of ¹³C atom), **6b** (280 mg, 99% of ¹³C atom), and **9b** (350 mg, 99% of ¹³C atom) were individually hydrolyzed in NaOH solution (10%, 3 mL) for 3 h at 100°C. The reaction mixtures were neutralized to pH 6 with 6 M HCl and cooled down in an ice bath. The precipitates were washed with ice-cold water six times (1 mL each) and dissolved in boiling methanol (SAs: methanol = 1: 1 / w: v). The methanol solutions were cooled in an ice bath to recrystallize the products, which were then separated by centrifugation (10 min, 2810 g) and washed twice with ice-cold methanol, giving **5b** (238 mg, 99.0% purity (**SI.4**)), **7b** (204 mg, 98.0% purity (**SI.4**)), and **10b** (276 mg, 98.0% purity (**SI.4**)) in 92.5%, 83.7%, and 91.7% yield, respectively.

Results And Discussion

Labelled SMX, SMM, and SDZ with uniformly labelling of ¹³C and ¹⁴C on the phenyl ring were prepared from commercially available labelled aniline, via four-step or five step syntheses (Fig. 1). The yields and radiochemical or chemical purities of the products are summarized in Table 1. Three unlabelled SAs and intermediates were synthesized in the same way and characterized by HPLC-Q-TOF-MS/MS and NMR, shown in **SI, Table S2**.

Table 1
Yields and purities of synthesized [¹⁴C]- or [¹³C]-labelled compounds.

Compounds	Label	Yield (%)	Purity (%)
2a	[¹⁴ C]	90.0	99.0 ^a
2b	[¹³ C]	95.7	99.0 ^b
3a	[¹⁴ C]	53.9 ^c	96.0 ^a
		74.3 ^d	93.0 ^a
3b	[¹³ C]	82.8	96.0 ^b
4a	[¹⁴ C]	51.0	95.0 ^a
4b	[¹³ C]	73.8	95.0 ^b
5a	[¹⁴ C]	90.9	98.1 ^e
5b	[¹³ C]	92.5	99.0 ^b
6a	[¹⁴ C]	15.6	95.0 ^a
6b	[¹³ C]	42.3	93.0 ^b
7a	[¹⁴ C]	66.2	98.3 ^e
7b	[¹³ C]	83.7	98.0 ^b
8a	[¹⁴ C]	98.3	99.0 ^a
8b	[¹³ C]	73.0	98.0 ^b
9a	[¹⁴ C]	36.0	57.0 ^a
9b	[¹³ C]	74.8	93.0 ^b
10a	[¹⁴ C]	79.9	98.3 ^e

^a Radiochemical purity was determined by TLC coupled with autoradiography

^b Chemical purity of [¹³C]-labelled intermediates and SAs was determined by HPLC.

^c **3a** was obtained after purification by flash column chromatography.

^d **3a** was obtained without purification.

^e Radiochemical purity was determined by HPLC coupled with LSC.

Compounds	Label	Yield (%)	Purity (%)
10b	[¹³ C]	91.7	98.0 ^b
^a Radiochemical purity was determined by TLC coupled with autoradiography			
^b Chemical purity of [¹³ C]-labelled intermediates and SAs was determined by HPLC.			
^c 3a was obtained after purification by flash column chromatography.			
^d 3a was obtained without purification.			
^e Radiochemical purity was determined by HPLC coupled with LSC.			

Synthesis of [¹⁴C]- or [¹³C]-SMX, [¹⁴C]- or [¹³C]-SMM, and [¹⁴C]- or [¹³C]-SDZ

Chlorosulfonation of aniline on the *para*-position of the amino group is the key step for the synthesis of SAs. Prior to the chlorosulfonation with ClSO₃H, acetylation of aniline is needed to prevent possible oxidation of the amino group and *bis*-sulfonation on the ring during chlorosulfonation. We performed the acetylation in aqueous solution with addition of K₂CO₃ to improve nucleophilic activity of aniline (**1a**), resulting in acetylaniline (**2a**) in a good yield of 90.0%. The method with less procedures was more convenient than the previous report [29].

Chlorosulfonation of **2a** with ClSO₃H generated the key intermediate **3a**, which can be used as precursor to synthesize a variety of [¹⁴C]-SAs with labeling on the phenyl ring by reacting with different amino heterocycles and subsequent alkaline hydrolysis. In a previous study, the synthesis of **3a** starting with 1.1 g of **2a**, and a high molar ratio of ClSO₃H was used together with **2a** (18:1) to obtain **3a** in the form of white solid after crystallizing in water [29]. However, the use of overdose of ClSO₃H was not applicable to the synthesis of **3a** at milligram scale (12 mg of **2a**) in our study, because the hot H₂SO₄, derived from hydrolysis of the excess ClSO₃H in water, could decompose **3a**, resulting in a low yield of **3a**. HPLC-Q-TOF-MS/MS analysis showed the conversion of large amount of **3a** to *N*-acetylsulfanilic acid in our synthesis (data not shown). In addition, solvent-free condition used in previously reported studies may result in inhomogeneous mixture of reactants at a micro-scale. Nguyen-Hoang-Nam et al. [28] also showed that it was difficult to synthesize sulfonyl chloride in a small amount and thus failed to obtain micro quantities (100 mg) of *N,N*-di(2-chloro-n-propyl)aminobenzenesulfonyl chloride labelled on the phenyl ring by chlorosulfonation with ClSO₃H and the corresponding [¹⁴C]-sulfonamide derivatives. In our synthesis, we used a low molar ratio of 1:7.4 in solvent CCl₄, and added NaCl to the reaction mixture to consume the by-product H₂SO₄. With these modifications on reaction conditions, we obtain **3a** in a good yield of 53.9% after purification (Table 1). Our method not only completely converted **2a**, but also reduced decomposition of **3a** by hot H₂SO₄, which was generated by hydrolysis of excess ClSO₃H.

Water inhibits the condensation of **3a** with amino heterocyclic compounds (*e.g.*, **11** and **12**). To avoid the water interference, molecular sieves were applied to adsorb the water during the condensation. With this method, we obtained **4a** and **6a** in good yield of 51.0% and 15.6%, respectively (Table 1).

The condensation of **3** with amino heterocycles was a nucleophilic substitution. Compound **11** had a higher nucleophilic activity than compound **12**, according to their electron cloud density, which was in agreement with the higher yield of **4a** than **6a** (51.0% vs. 15.6%, respectively) and **4b** than **6b** (73.8% vs. 42.3%, respectively) (Table 1). Condensation of **3** with other heterocyclic compounds could be used to prepare other [¹⁴C]- or [¹³C]-labelled sulfonamides, such as with 2-aminopyrimidine for SDZ [28]. However, owing to the low nucleophilic activity of 2-aminopyrimidine, the yield of **10a** at micro-scale was very low (7.4%, and overall yield of from **1a** to **10a** was 2.4%) and the yield of **9b** was also lower than **4b** and **6b** (21.0% vs. 73.8% and 42.3%, respectively) (Table 1). Therefore, for the preparation of **10a** and **10b**, we used a five-step synthetic pathway (Fig. 1). We used two steps to synthesize **9** instead of one step. We firstly synthesized **8** by condensation of **3** with ammonium hydroxide, which has a high nucleophilic activity and is a base capable of neutralizing the by-product H₂SO₄, in good yield of 98.3% for **8a** and 73.0% yield for **8b**. Coupling of **8** to **13** gave both **9a** and **9b** in good yield (36.0% and 74.8%, respectively). The synthesis of **9** from **3** via this two-step pathway not only completely converted **3** to **8** with a higher stability, to avoid the decomposition of **3**, but also gave a much higher overall yield than the one-step reaction (35.4% vs. 7.4% for **9a**, 54.6% vs. 21.0% for **9b**).

¹³C-NMR data of [¹³C]-SMX, [¹³C]-SMM and [¹³C]-SDZ

The ¹³C-NMR spectra of three [¹³C]-SAs and their corresponding unlabelled compounds were shown in Fig. 2. The significant triplet signals allow the assignment of a group of signals (*i.e.*, 112.53–112.98 ppm, 124.46–125.08 ppm, 129.16–130.16 ppm, and 153.25–153.51 ppm) to ¹³C-atoms of benzene ring. ¹³C-tracers could provide more structural information about fate and behaviors of labelled C-atoms in environmental matrixes than radioactive [¹⁴C]-tracer [30]. The peaks of C-atoms in ¹³C-labelled compounds are split into triplets due to ¹³C–¹³C coupling and have much higher intensity than those in the non-labelled compound with natural ¹³C-atom abundance (1.1%), thus the triplet signals can be used to identify chemical nature of labelled carbon atoms, *e.g.*, the residues of pesticides (*e.g.*, cyprodinil), humus monomers (*e.g.*, catechol), and emerging pollutants (*e.g.*, tetrabromobisphenol A) bound to soil humic substances [31–33], which provide more clear information about incorporation into humic substances of pollutants with labeling on single or double carbon atoms (*e.g.*, SDZ, nonylphenol, chlorophenol) [34–36].

Characteristics of the synthetic methods

The main advantages of our synthetic methods over those previously reported are the success to synthesize [¹⁴C]-SAs on micro-scale from commercially relatively cheap [¹⁴C]-labelled **1** (about 30.9 mg). Different from the classic synthetic pathway via condensation of **3** with aminoheterocycles, the new

pathway via condensation of **8** with chloroheterocycles is optimized for synthesis of [¹⁴C]-labelled SAs with an aminoheterocycle of low nucleophilic activity or high steric hinderance. For synthesis of [¹³C]-labelled SAs, both pathways provided good yields.

Purification of products is important for product quality. We also provide feasible methods for purification of small amount of [¹⁴C]-products and obtained [¹⁴C]-compounds with high purity. Crystallization in water as purification procedure or direct use of reaction mixture of previous synthetic step without further purification could be applicable to synthesis of unlabelled SAs at gram-scale [26, 27], which are however not applicable to the synthesis of [¹⁴C]-labelled SAs at milligram-scale, because recrystallization may recover much less products. In this study, we used classic chromatographic separation method, such as flash column chromatography and preparative TLC, to purify small amount of [¹⁴C]-products.

Conclusions

This study describes optimized methods for synthesis of SAs labelled with ¹⁴C or ¹³C on the phenyl ring from commercially available [¹⁴C]- or [¹³C]-aniline, especially the synthesis of [¹⁴C]-labelled SAs on a micro-scale (milligram-level). Three typical sulfonamide antibiotics SMX, SMM, and SDZ with [¹⁴C]- or [¹³C]-labelling prepared in good yields (totally 5.0 ~ 22.5% for ¹⁴C, 28.1 ~ 54.1% for ¹³C, relatively to aniline). The methods consist of four-step (via condensation of **3** and aminoheterocycles) or five-step (via condensation of **8** and chloroheterocycles) reactions. The four-step pathway is suitable for synthesis of large amount of SAs (*e.g.*, gram level) or SAs containing aminoheterocycles of high nucleophilic activity, while the five-step pathway is especially applicable to synthesis of SAs (*e.g.*, SDZ) at milligram scale containing an aminoheterocycle of low nucleophilic activity. This study provided synthetic methods for effective laboratory preparation of commercially unavailable labelled SAs, which benefit to studies on fate and behavior of SAs in both natural and engineered environments and biological systems.

Abbreviations

- SAs
- sulfonamide antibiotics
- SMX
- sulfamethoxazole, SMM:sulfamonometh-oxine
- SDZ
- sulfadiazine
- NERs
- non-extractable residues
- NMR
- nuclear magnetic resonance
- TLC
- thin-layer chromatography

HPLC

high-performance liquid chromatography

LSC

liquid scintillation counting

MS

mass spectrometer

R_f

retardation factor

t_R

retention time.

Declarations

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Authors' contributions

XW conceptualized and designed experiment, analyzed the data, wrote, revised the manuscript. YY assisted in experiment design and materials. LW and DZ supported the analysis of HPLC and HPLC-Q-TOF-MS/MS, experiment design and revised the manuscript. FS directed the study, analyzed the data, revised and finalized the manuscript. JC supported the study, assisted in planning of experiments and experimental equipment. RJ and PC provided test funds, and directed the study, revised the manuscript. All the authors read and approved the final manuscript.

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Availability of data and materials

The complete dataset of this study is included within the article and the Supporting Information.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

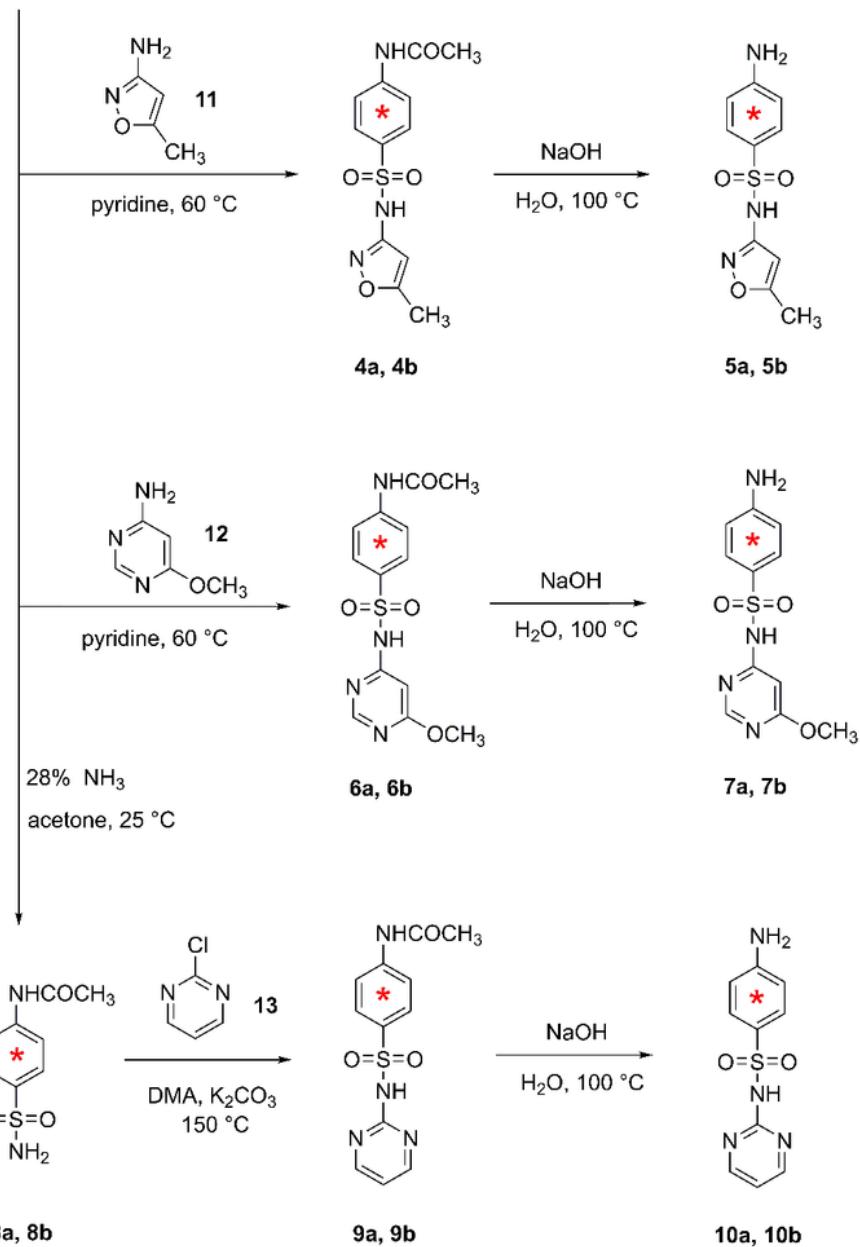
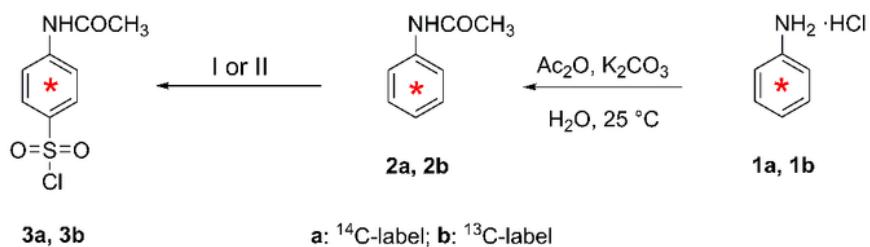


Figure 1

Synthetic pathways of [¹⁴C]- and [¹³C]-labelled SMX (5a, 5b), [¹⁴C]- and [¹³C]-labelled SMM (7a, 7b) and [¹⁴C]- and [¹³C]-labelled SDZ (10a, 10b). (I) Method: CISO₃H + NaCl in CCl₄, 58 °C; (II) Method: CISO₃H + SOCl₂ in CCl₄, 58 °C.

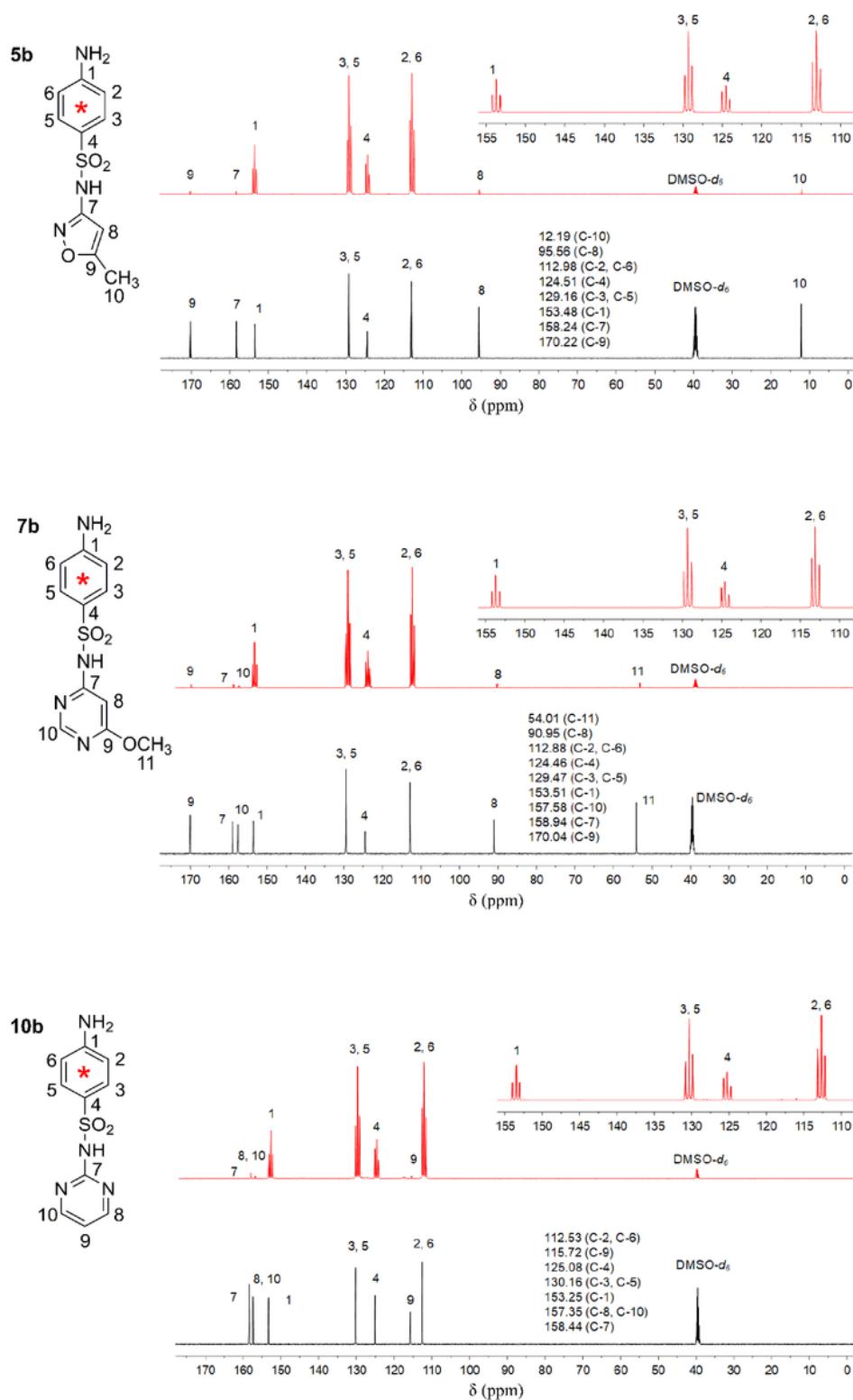


Figure 2

13C-NMR spectra of [13C]-SMX (5b), [13C]-SMM (7b), and [13C]-SDZ (10b). Positions of the numbered C-atoms are given in the corresponding structure of [13C]-labelled SAs. The red and black lines represent spectra of SAs with [13C]-labelling and natural 13C-abundance. The enlarged figures of signal of the C-atoms at [13C]-labelled benzene ring is shown. Chemical shifts of numbered C-atoms with natural abundance shown in the chemical structures are listed.

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