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Chiral Analysis of the Key Intermediates of Tenofovir Alafenamide Fumarate

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Abstract

(*R*)-Tenofovir phenyl ester ((*R*)-**1**) and (*R*)-tenofovir diphenyl ester ((*R*)-**2**) are key intermediates for the practical synthesis of tenofovir alafenamide fumarate, which is a mainstay antiretroviral for the treatment of chronic hepatitis B and HIV-1 infections. This article deals with the chiral analysis of (*R*)-**1** and (*R*)-**2** against their respective optical impurity (*S*)-tenofovir phenyl ester ((*S*)-**1**) and (*S*)-tenofovir diphenyl ester ((*S*)-**2**) using a polysaccharide-coated chiral stationary phase (CSP) by normal-phase high-performance liquid chromatography (HPLC). To this end, a chiral synthetic strategy for (*S*)-**2** was efficiently executed capitalizing on a classical Mitsunobu reaction to stereospecifically invert the configuration of chiral carbon in readily accessible (*R*)-HPA ((*R*)-**4**) to deliver (*S*)-HPA ((*S*)-**4**), from which (*S*)–tenofovir ((S)-**3**) was in turn prepared and further transformed into (*S*)-**2**. With reference substance (*S*)-**2** in hand, a chiral analytical method for (*R*)-**2** using Chiralpak AD-H as CSP by normal-phase HPLC has been developed and validated. The validation results indicated that this chiral analytical method has been achieved with satisfactory separation effect, high sensitivity, and good precision and accuracy, and thus can be deployed for the determination of optical impurities in samples of (*R*)-**1** (via derivation to (*R*)-**2**) and (*R*)-**2**.

Keywords
- tenofovir alafenamide fumarate
- optical impurity
- Mitsunobu reaction
- reference substances
- chiral stationary phase

Introduction

Tenofovir alafenamide fumarate (TAF) is an oral phosphonamidate prodrug of antiretroviral tenofovir (PMPA) for the treatment of patients with chronic hepatitis B virus infection as monotherapy, and for the treatment of patients with HIV-1 infection in fixed-dose combinations. TAF has greater stability in plasma compared with its congener tenofovir disoproxil fumarate (TDF). Switching from TDF to TAF maintained or improved virological and biochemical responses with improved bone and renal safety.[1–3]

Stereoisomers introduced into the human body could exhibit different pharmacokinetic, pharmacodynamic, and toxicologic profiles.[4] Therefore, chiral purity control of optically active drug substances is essential for the integrity and quality of related drug products. TAF is a chiral drug chemically belonging to acyclic nucleoside phosphonates. There are two chiral carbons and one chiral phosphorus in the molecular structure of its base, i.e., tenofovir alafenamide. (*R*)-Tenofovir phenyl ester ((*R*)-**1**)[5,6] and (*R*)-tenofovir diphenyl ester ((*R*)-**2**)[7,8] can all serve as key intermediates in the final steps in the practical synthesis of TAF (►**Fig. 1**). They all carry a chiral carbon of (*R*) configuration, which in turn stems from PMPA. So far, there are mainly three synthetic strategies for the construction of this (*R*)-carbon in PMPA. First, the desired chirality was imported from readily available (*R*)-propylene carbonate as the chiral pool.[9,10] Second, the desired chirality arrived in 97% *ee* (enantiomeric excess) via regioselective and

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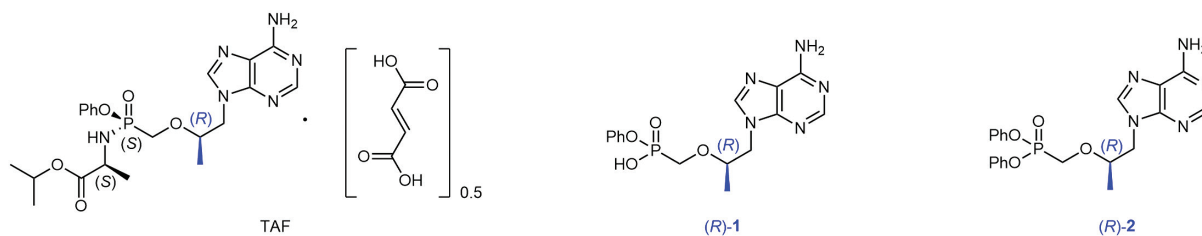


Fig. 1 TAF and key intermediates (*R*)-**1** and (*R*)-**2** thereof. TAF, tenofovir alafenamide fumarate.

enantioselective kinetic resolution of racemic propylene oxide with $TMSN_3$ in the presence of (salen)CrN_3 complex.[11] Third, the desired chirality was synthesized in 96% *ee* through asymmetric transfer hydrogenation of achiral purine derivative.[12] So chiral impurity with (*S*)-carbon in (*R*)-**1** and (*R*)-**2** could come from the chiral pool, kinetic resolution, and asymmetric synthesis.

Since (*R*)-**1** and (*R*)-**2** are all advanced intermediates in the practical synthesis of TAF, their chiral purity needs to be controlled for the sake of purity and yield of TAF. To the best of our knowledge and our surprise, there has been no reported documentation of chiral analysis of (*R*)-**1** and (*R*)-**2** yet. In a related field, the preparation method of TAF enantiomer has been disclosed,[13] and the chiral stationary phase (CSP) high-performance liquid chromatography (HPLC) method for the separation of TAF and its enantiomer has been reported.[14] Our initial scouting experiments showed that (*R*)-**1** and (*S*)-tenofovir phenyl ester ((*S*)-**1**) can be neither separated on a CSP by reversed-phase HPLC (RP-HPLC) nor separated with acceptable peak shape and separation effect on CSP by normal-phase HPLC (NP-HPLC). Fortunately, (*R*)-**1** and (*S*)-**1** can be reliably transformed into (*R*)-**2** and (*S*)-**2**, respectively, with their chirality preserved. Therefore, this study aimed at developing and validating a chiral HPLC method for the determination of the enantiomer impurity (*S*)-**2** in (*R*)-**2** samples at a relative concentration of 0.1% with acceptable precision and accuracy.

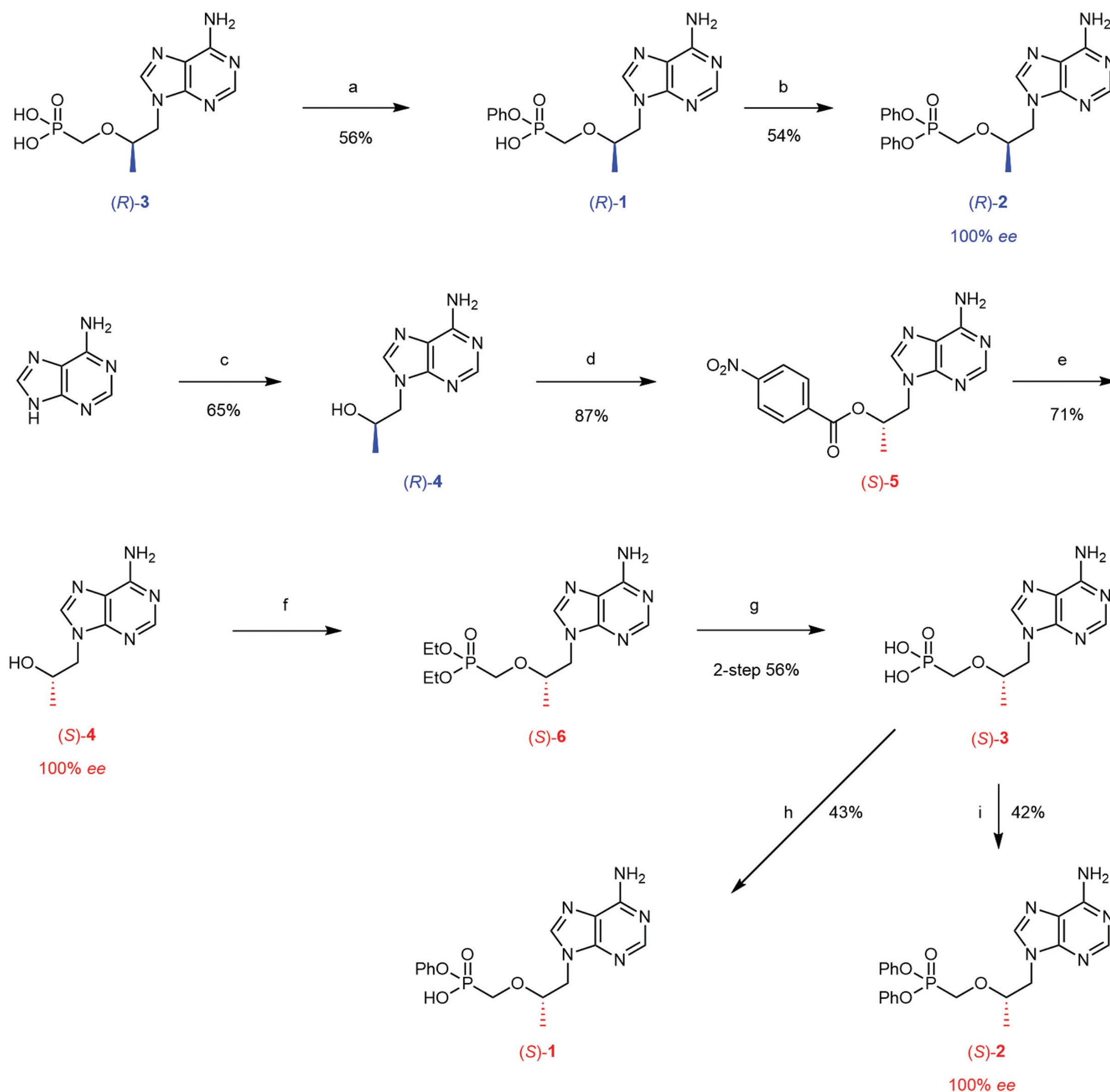
Results and Discussion

Synthesis of Reference Substances

Given the availability of (*R*)-tenofovir ((*R*)-**3**) and bulk chiral pool of (*R*)-propylene carbonate at hand and our extensive experience in the Mitsunobu reaction, we set out to explore an efficient synthesis of enantiopure reference substances for the development of the above-mentioned chiral analysis, though using racemic reference substances is also a scientifically sound option. (*R*)-**1** and (*S*)-**1** were prepared from (*R*)-**3** and (*S*)-tenofovir ((*S*)-**3**) as reported in the patent literature,[5] respectively. (*R*)-**2** was prepared from (*R*)-**1** as described in the patent literature.[15] (*S*)-tenofovir diphenyl ester ((*S*)-**2**) was prepared from (*S*)-**3** via reaction with thionyl chloride to afford a dichloride intermediate, followed by esterification with phenol.[8] Preparation of (*S*)-**3** is as follows: (*R*)-9-(2-hydroxypropyl)adenine ((*R*)-HPA, (*R*)-**4**) was accessed via alkylation of adenine with (*R*)-propylene carbonate following the routine procedure.[9] The Mitsunobu reaction of (*R*)-**4** with 4-nitrobenzoic acid in the presence of triphenyl phosphine and diisopropyl azodicarboxylate (DIAD) gave ester (*S*)-**5**.[16,17] (*S*)-**5** was treated with sodium methoxide in methanol to deliver (*S*)-HPA ((*S*)-**4**). (*S*)-**4** was then *O*-alkylated with tosylated hydroxymethylphosphonate diester (DESMP) in the presence of magnesium *tert*-butoxide (MTB) to deliver diethyl phosphonate (*S*)-**6**, which was treated with sodium bromide and trimethylsilyl chloride combination for diethyl removal to finally deliver (*S*)-**3**. The synthetic routes are outlined in ►**Scheme 1**.

(*S*)-**3** is the key material for the synthesis of both (*S*)-**1** and (*S*)-**2**. Holy's group has reported a stepwise approach[18] as well as a synthon one[19] for the preparation of (*S*)-**3**. These two approaches all take ethyl *D*-(+)-lactate as the enantiopure starting material, which needs two additional steps of hydroxyl protection and ester reduction to be transformed into a useful form. Further, the stepwise approach suffers from frequent use of protective groups and lengthy reaction procedures, while the synthon approach suffers from the use of either chloromethylation or sodium hydride and the dependency on silica gel chromatography or ion exchange chromatography for the purification of all the intermediates and product. To address these shortcomings, a new synthetic route was elaborated using bulk (*R*)-propylene carbonate as the chiral pool, and capitalizing on a classical Mitsunobu reaction to stereospecifically invert the configuration of the chiral carbon to yield the desired (*S*) chirality. Thus, (*S*)-**3** was efficiently accessed in five steps from adenine and (*R*)-propylene carbonate (►**Scheme 1**). Our synthesis of (*S*)-**3** boasts bulk chiral pool, reliable reactions and no chromatography.

Krait et al has reported a capillary electrophoresis method for the assessment of the chiral purity of (*R*)-**3** against (*S*)-**3**.[20] Alternatively, we have developed a high-resolution (8.00) NP-HPLC method using Daicel's Chiralpak AD-H as CSP with (*R*)-**4** eluting at 11.308 minutes and (*S*)-**4** eluting at 15.708 minutes (►**Fig. 2**). The chiral purity of the obtained (*S*)-**4** was checked thereby with the result suggesting a complete inversion of chiral carbon configuration by the Mitsunobu reaction as expected. The proton NMR spectrum of the obtained (*S*)-**4** is identical to that of (*R*)-**4**. Taken together, the structure and chirality of (*S*)-**4** have been elucidated. (*S*)-**3** was in turn prepared from (*S*)-**4** as per the established synthetic procedures from (*R*)-**4** to (*R*)-**3** without interfering with the chiral carbon.[9] The proton NMR spectrum of obtained (*S*)-**3** is identical to that of authentic (*R*)-**3**. Thus, the structure and chirality of (*S*)-**3** have been well established based on these available data.



Scheme 1 Synthetic routes to compounds (*R*)-**1**, (*R*)-**2**, (*S*)-**1**, and (*S*)-**2**. *Reagents and conditions*: (**a**) $P(OPh)_3$, Et_3N, DMAP, CH_3CN, 80°C, 48 hours; (**b**) (1) $SOCl_2$, CH_3CN, reflux, 3 hours; (2) PhOH, Et_3N, –25°C, 1 hour; (**c**) (*R*)-propylene carbonate, NaOH, DMF, 120°C, 24 hours; (**d**) 4-nitrobenzoic acid, PPh_3, DIAD, DMF, 30°C, 2.5 hours; (**e**) MeONa, MeOH, r.t., 2.5 hours; (**f**) DESMP, MTB, NMP, 75°C, 3 hours; (**g**) NaBr, TMSCl, 75°C, 4 hours; (**h**) $P(OPh)_3$, Et_3N, DMAP, CH_3CN, 80°C, 48 hours, (**i**) (1) $SOCl_2$, CH_3CN, reflux, 3 hours; (2) PhOH, Et_3N, –30°C, 2.5 hours.

Development and Validation of the Analytical Method

A screening study of the chiral HPLC method for separating the enantiomeric pair of (*R*)-**2** and (*S*)-**2** was undertaken on CSP from Daicel based upon silica gel coated with polysaccharide derivative (►**Table 1**).[21] Altogether four kinds of Daicel CSPs have been screened, including Chiralcel OD-H based upon silica gel coated with cellulose tris(3,5-dimethylphenylcarbamate), Chiralpak AD-H based upon silica gel coated with amylose tris(3,5-dimethylphenylcarbamate), Chiralpak AS-H based upon silica gel coated with amylose tris[(*S*)-α-methylbenzylcarbamate], and Chiralpak AY-H based upon silica gel coated with amylose tris(5-chloro-2-methylphenylcarbamate). Addition of diethylamine in the mobile phase was found to be beneficial to improving the peak shape and separation effect. Screening results indicated that the enantiomers were resolved with satisfactory resolutions (>5) and good peak shape on Chiralpak AD-H (entries 4 and 5) and Chiralpak AY-H (entry 8), while Chiralcel OD-H delivered a double-headed peak without baseline separation (entries 1–3), and Chiralpak AS-H delivered a single peak (entry 7). Ethanol acting as solution B in the mobile phase proved to be detrimental to resolution (entry 6). Considering the issues of column pressure (entry 5) and column availability (entry 8), HPLC conditions using Chiralpak AD-H in entry 4 were selected for analytical method validation. A typical chromatogram for entry 4 is presented in ►**Fig. 3** with (*S*)-**2** eluting at 17.731 minutes and (*R*)-**2** eluting at 23.898 minutes.

The validation data indicated that the linear correlation coefficients of (*R*)-**2** and (*S*)-**2** are all more than 0.999,

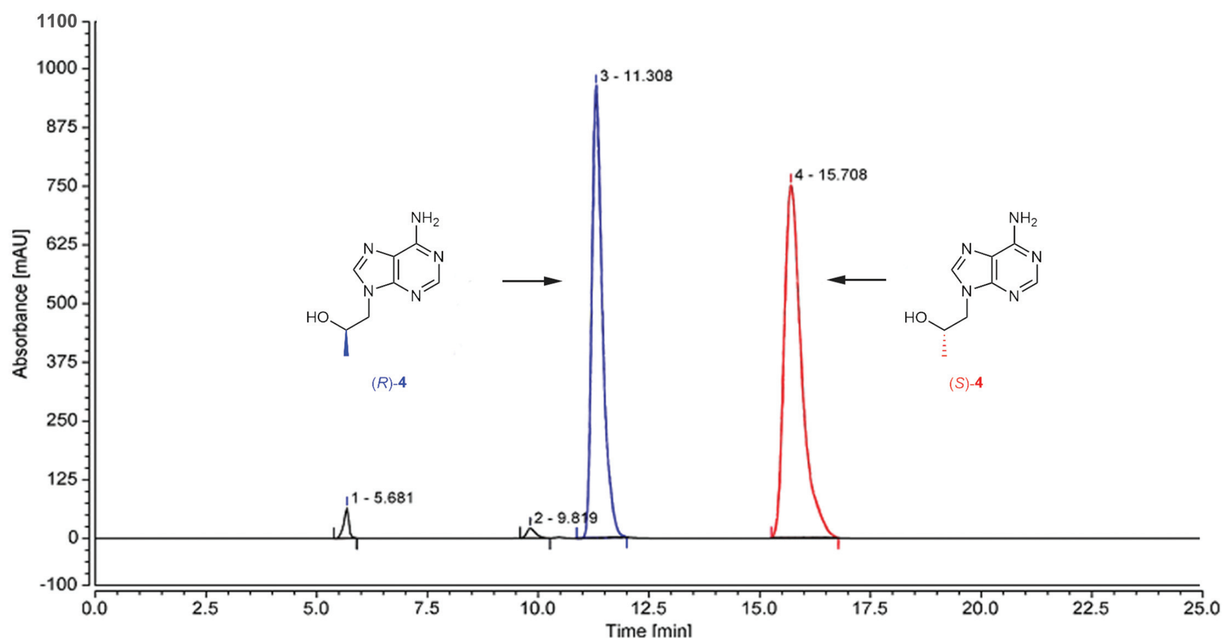


Fig. 2 A typical chromatogram separating (*R*)-**4** and (*S*)-**4**.

Table 1 Screening of HPLC conditions for separating (*R*)-2 and (*S*)-2[a]

Entry	CSP[b]	Mobile phase[c]	Flow rate (mL/min)	Resolution	Peak width[d] (min)	Retention time[d] (min)
1	OD-H	I, 70:30	0.6	0	(*S*): 1.09	(*S*): 15.726
					(*R*): 1.30	(*R*): 16.431
2	OD-H	II, 70:30	0.6	0	(*S*): 1.10	(*S*): 16.438
					(*R*): 1.31	(*R*): 17.233
3	OD-H	I, 50:50	0.6	0	(*S*): 0.57	(*S*): 10.061
					(*R*): 0.68	(*R*): 10.594
4	AD-H	I, 50:50	0.6	5.80	(*S*): 0.89	(*S*): 17.731
					(*R*): 1.26	(*R*): 23.898
5	AD-H	I, 40:60	0.5	5.96	(*S*): 0.87	(*S*): 17.999
					(*R*): 1.25	(*R*): 24.246
6	AD-H	III, 40:60	0.5	1.94	(*S*): 1.93	(*S*): 11.703
					(*R*): 2.09	(*R*): 15.536
7	AS-H	I, 50:50	0.6	0	0.72	11.123
8	AY-H	I, 50:50	0.6	11.72	(*S*): 0.67	(*S*): 14.441
					(*R*): 1.23	(*R*): 25.501

[a]HPLC analysis was performed on Dionex UltiMate 3000 System. Detector: UV 260 nm, column temperature: 35°C, injection volume: 10 μL, isocratic elution for 30 minutes.
[b]OD-H: Chiralpak OD-H (4.6 mm × 250 mm, 5 μm), AD-H: Chiralpak AD-H (4.6 mm × 250 mm, 5 μm), AS-H: Chiralpak AS-H (4.6 mm × 250 mm, 5 μm), AY-H: Chiralpak AY-H (4.6 mm × 250 mm, 5 μm).
[c]Mobile phase I: solution A, *n*-hexane (0.1% diethylamine); solution B, isopropanol (0.1% diethylamine), variant volume ratios. Mobile phase II: solution A, *n*-hexane; solution B, isopropanol. Mobile phase III: solution A, *n*-hexane (0.1% diethylamine); solution B, ethanol (0.1% diethylamine). Ratios are volume ratios.
[d](*R*) for (*R*)-**2**, (*S*) for (*S*)-**2**.

and LOD and LOQ of them all meet the requirements of the 0.1% control threshold. The relative standard deviations (RSDs) ($n=6$) of retention time and peak area of (*R*)-**2** and (*S*)-**2** are within the range of 0.04–0.11% and 0.54–0.98%, respectively, so that the method can be considered precise. The recoveries ($n=9$) are within the range of 96.0 to 104% so that the method can be considered accurate.

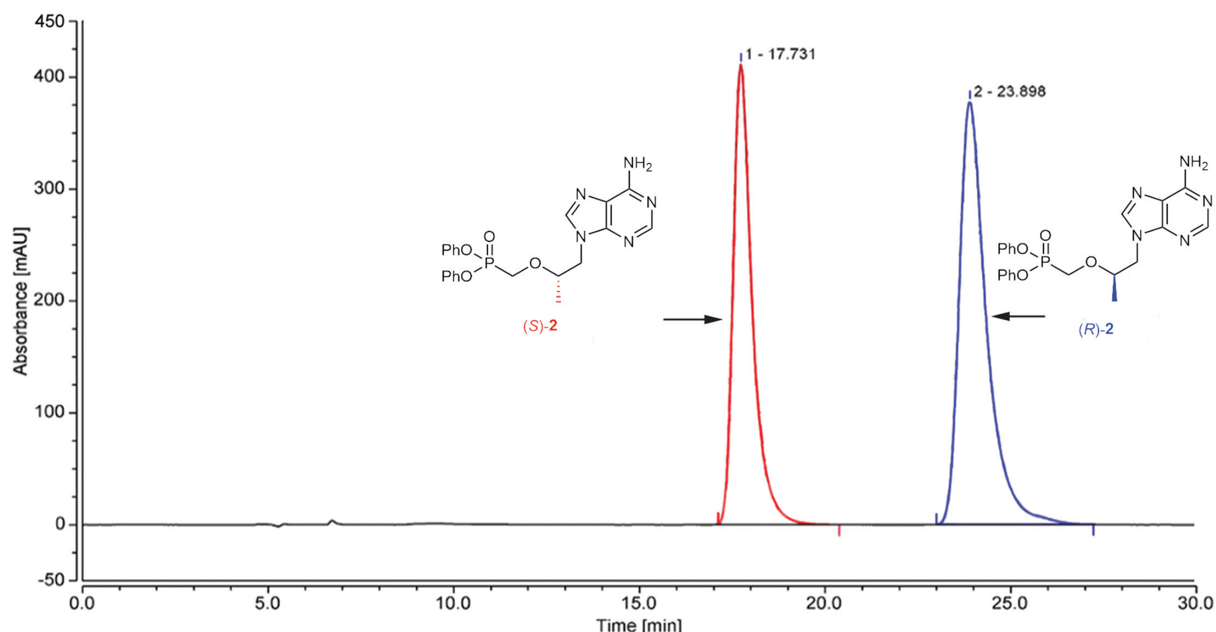


Fig. 3 A typical chromatogram separating (*R*)-**2** and (*S*)-**2** in entry 4, Table 1.

Conclusion

A NP-HPLC method using polysaccharide-derivative-based CSP has been developed and validated for the determination of the optical purity of (*R*)-**1** (via derivation to (*R*)-**2**) and (*R*)-**2**, two key intermediates for the practical synthesis of TAF. In the preparation of reference substance (*S*)-**2**, an efficient synthesis of (*S*)-**3** has been accomplished by capitalizing on the classical Mitsunobu reaction to construct the desired (*S*) chirality from the bulk chiral pool. (*S*)-**2** and intermediate (*S*)-**5** are new compounds that have not been reported in the literature. The developed method has high sensitivity as well as good precision and accuracy so that it can be deployed for the determination of optical impurities in the samples of key TAF intermediates (*R*)-**1** (via derivation) and (*R*)-**2**.

Experimental Section

Chemical Synthesis

All solvents and reagents were obtained from commercial suppliers and used without further purification unless otherwise noted. Reaction progress was monitored by thin layer chromatography (TLC) using precoated glass-backed silica gel plates and visualized with UV detection at 254 nm. Silica gel 200–300 mesh was employed for column chromatography purification. Solvent ratios are volume ratios. Evaporation of solvents was performed on a rotary evaporator under reduced pressure. ^{1}H NMR, ^{13}C NMR, and ^{31}P spectra were recorded at ambient temperature on either a Bruker Avance III 400 or Bruker Avance Neo 600 spectrometer. NMR spectra were obtained in either deuterated dimethyl sulfoxide (DMSO-d_6) or deuterium oxide (D_2O). ^{1}H NMR and ^{13}C NMR spectra were referenced to the residual solvent peak. ^{31}P NMR spectra in DMSO-d_6 were referenced to an external standard of 85% phosphoric acid (δ 0.00 ppm). Chemical shifts are given in δ values and coupling constants are reported in hertz (Hz). Melting points were determined on a TIANDA TIANFA YRT-3 melting apparatus and were uncorrected. Optical rotations were measured on an Anton Paar MCP500 polarimeter at 20°C.

HPLC analyses were performed on Dionex UltiMate 3000 System. NP-HPLC analysis of (*R*)-**4** and (*S*)-**4**: Daicel Chiralpak AD-H (4.6 mm × 250 mm, 5 µm), isocratic elution (A, *n*-hexane (0.1% diethylamine); B, ethanol (0.1% diethylamine); B 50%) for 25 minutes, flow rate: 0.6 mL/min, detector: UV 266 nm, column temperature: 35°C, injection volume: 5 µL. Sample solutions of 1 mg/mL were prepared with ethanol. RP-HPLC analysis of (*R*)-**1** and (*S*)-**1**: NanoChrom ChromCore 120 C18 (4.6 mm × 250 mm, 5 µm), gradient elution (A, 10 mmol/L aqueous ammonium formate (adjusted to pH 3 with formic acid); B, methanol; 0–8 minutes: B 10–10%, 9–35 minutes: B 10–90%, 36–37 minutes: B 90%, 38–40 minutes: B 90–10%, 41–45 minutes: B 10%), flow rate: 1 mL/min, detector: UV 260 nm, column temperature: 30°C, injection volume: 5 µL. Sample solutions of 2 mg/mL were prepared with acetonitrile. RP-HPLC analysis of (*R*)-**2** and (*S*)-**2**: NanoChrom ChromCore 120 C18 (4.6 mm × 250 mm, 5 µm), gradient elution (A, 10 mmol/L aqueous ammonium formate (adjusted to pH 3 with formic acid); B, acetonitrile; 0–25 minutes: B 10–90%, 25–28 minutes: B 90%, 28–32 minutes: B 90–10%, 32–38 minutes: B 10%), flow rate: 1 mL/min, detector: UV 260 nm, column temperature: 35°C, injection volume: 5 µL. Sample solutions of 2 mg/mL were prepared with acetonitrile. RP-HPLC purity was calculated through % area normalization.

Phenyl Hydrogen ((((*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate ((*R*)-1)

To a stirring mixture of (*R*)-**3** (10.0 g, 34.8 mmol) and sieve-dried acetonitrile (80 mL) in a 250 mL three-necked round-bottomed flask under nitrogen was added sequentially triethylamine (7.05 g, 69.6 mmol), 4-dimethylaminopyridine

(DMAP) (4.25 g, 34.8 mmol), and triphenyl phosphite (16.2 g, 52.22 mmol). The reaction mixture was heated and stirred at 80°C for 48 hours when the reaction was judged complete by TLC (CH_2Cl_2:MeOH = 1:1, one drop of triethylamine). Then the solvents were evaporated under reduced pressure (60°C) to afford a yellow oil residue, which was partitioned between ethyl acetate (20 mL) and water (30 mL). The aqueous layer was collected, washed with ethyl acetate (20 mL ×2), and then the pH was adjusted to 3 with 37% aqueous HCl to initiate crystallization. The resulting slurry was stirred at 30°C for 30 minutes. After the pH was further adjusted to 2 with 37% aqueous HCl, the slurry was stirred at 20°C for 30 minutes, 10°C for 2 hours, and then filtered. The filter was washed with cold pH 2 aqueous HCl (20 mL), and dried in vacuo (45°C, 4 hours) to afford (*R*)-**1** as a white solid (7.05 g, 56% yield, RP-HPLC purity: 98.36%). ^{1}H NMR (400 MHz, D_2O) *δ* 8.35 (s, 1H), 8.27 (s, 1H), 7.26 (t, *J* = 7.9 Hz, 2H), 7.18–7.09 (m, 1H), 6.76 (d, *J* = 8.7 Hz, 2H), 4.46 (dd, *J* = 14.8, 2.9 Hz, 1H), 4.30 (dd, *J* = 14.8, 8.9 Hz, 1H), 4.15–4.07 (m, 1H), 3.86 (dd, *J* = 13.7, 7.8 Hz, 1H), 3.60 (dd, *J* = 13.8, 9.0 Hz, 1H), 1.32 (d, *J* = 6.3 Hz, 3H). mp: 229.1–231.7°C, which was consistent with a reported study (mp: 221.1–223.0°C).[22] $[\alpha]_{589}^{20}$ −3.7° (*c* = 1, 0.03 mol/L aqueous NaOH).

Diphenyl (*R*)-(((1-(6-amino-9*H*-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate ((*R*)-2)

To a stirring mixture of (*R*)-**1** (2.00 g, 5.5 mmol) and sieve-dried acetonitrile (10 mL) in a 50 mL three-necked round-bottomed flask was added thionyl chloride (1.31 g, 11.0 mmol) at ambient temperature. The reaction mixture was heated and stirred under reflux for 3 hours. The resulting mixture was evaporated under reduced pressure to deliver a yellow oil residue, which was dispersed in sieve-dried acetonitrile (10 mL). The dispersion was cooled to −25°C, and phenol (1.04 g, 11.0 mmol) was added followed by a dropwise addition of triethylamine (*ca* 2 mL) to adjust the pH to 7–8. The resulting mixture was stirred at −25°C under nitrogen for 1 hour and then evaporated under reduced pressure to deliver an off-white solid residue, which was purified on a silica gel column (EtOAc:MeOH = 100:1 →50:1) to deliver (*R*)-**2** as a white solid (1.3 g, 54% yield, RP-HPLC purity: 97.28%). ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 8.13 (s, 1H), 8.04 (s, 1H), 7.42–7.31 (m, 4H), 7.27–7.17 (m, 4H), 7.12 (d, *J* = 8.3 Hz, 2H), 7.06 (d, *J* = 8.3 Hz, 2H), 4.35–4.12 (m, 4H), 4.13–4.01 (m, 1H), 1.13 (d, *J* = 6.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) *δ* 155.98, 152.43, 149.80, 149.64 (d, *J* = 5.6 Hz), 149.55 (d, *J* = 5.6 Hz), 141.30, 129.92 (d, *J* = 4.1 Hz), 125.40, 125.36, 120.53 (d, *J* = 4.0 Hz), 120.46 (d, *J* = 4.0 Hz), 118.43, 75.74 (d, *J* = 13.3 Hz), 61.24 (d, *J* = 166.0 Hz), 46.83, 16.62. ^{31}P NMR (162 MHz, DMSO-d_6) *δ* 16.03. mp: 132.8–133.1°C, which was consistent with a reported study (mp: 131.7–132.7°C).[22]

(*S*)-(((1-(6-amino-9*H*-purin-9-yl)propan-2-yl)oxy)methyl)phosphonic acid ((*S*)-3)

(*S*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl 4-nitrobenzoate ((*S*)-5)

To a stirring mixture of (*R*)-**4** (20 g, 103.5 mmol), 4-nitrobenzoic acid (34.6 g, 207 mmol), and triphenylphosphine (54.3 g, 207 mmol) in sieve-dried DMF (300 mL) in a 1 L three-necked round-bottomed flask was added at 0°C under nitrogen DIAD (41.9 g, 207 mmol). The mixture was then warmed to ambient temperature (30°C) and stirred for 2.5 hours. The resulting slurry was filtered. The filter was washed with saturated aqueous sodium bicarbonate followed by ethyl acetate, and dried in vacuo (60°C, 5 hours) to furnish crude (*S*)-**5** as a yellowish green solid (31 g, 87% yield). This crude (*S*)-**5** was used in the next step without further purification.

Another batch of crude (*S*)-**5** prepared with the same procedure was purified on a silica gel column (EtOAc:MeOH = 20:1) to furnish pure (*S*)-**5** as a pale yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) *δ* 8.30 (d, *J* = 8.8 Hz, 2H), 8.20 (s, 1H), 8.10 (s, 1H), 8.10 (d, *J* = 8.6 Hz, 2H), 7.29 (s, 2H), 5.50–5.43 (m, 1H), 4.54 (dd, *J* = 14.7, 3.3 Hz, 1H), 4.48 (dd, *J* = 14.7, 7.3 Hz, 1H), 1.36 (d, *J* = 6.4 Hz, 3H).

(*S*)-9-(2-hydroxypropyl)adenine ((*S*)-4)

Crude (*S*)-**5** (31 g, 90.6 mmol) obtained above was dissolved in methanol (465 mL), and a solution of sodium methoxide (0.49 g, 9.1 mmol) in methanol (2 mL) was added at ambient temperature. The mixture was then stirred at ambient temperature for 2.5 hours. The resulting reaction mixture was evaporated under reduced pressure to give a pale yellow solid residue, which was slurried in ethyl acetate (500 mL). The slurry was filtered. The filter was rinsed with ethyl acetate (100 mL), and then dried in vacuo (60°C, 5 hours) to give a crude product. Crude (*S*)-**4** was re-slurried in a mixture of methanol and isopropanol (1:1) (20 mL) to give after filtration and drying pure (*S*)-**4** as a white solid (12.5 g, 71% yield). ^{1}H NMR (600 MHz, DMSO-d_6) *δ* 8.13 (s, 1H), 8.04 (s, 1H), 7.18 (s, 2H), 5.05 (br s, 1H), 4.12–4.06 (m, 1H), 4.04–3.97 (m, 2H), 1.05 (d, *J* = 5.9 Hz, 3H).

(S)-tenofovir ((S)-3)

To a stirring mixture of (*S*)-**4** (4 g, 20.7 mmol) and MTB (5.3 g, 31.1 mmol) in sieve-dried NMP (20 mL) was added dropwise at 70°C under nitrogen DESMP (10 g, 31.0 mmol) in 15 minutes. The mixture was heated and stirred at 75°C for 3 hours, and then cooled to room temperature. After the pH was adjusted to 6 with acetic acid, the resulting mixture was poured into ethyl acetate (100 mL) at 50 to 60°C and stirred at the same temperature for 30 minutes. The resulting slurry was filtered to remove precipitated magnesium salts. The filter was extracted with ethyl acetate (100 mL) at 50 to 60°C under stirring for 30 minutes, and filtered. The combined filtration was evaporated under reduced pressure to give crude (*S*)-**6** as a yellow oil residue.

To a stirring mixture of the above-obtained crude (*S*)-**6** and sodium bromide (7.5 g, 72.5 mmol) at 0°C under nitrogen was added trimethylsilyl chloride (12 g, 109.7 mmol) dropwise with temperature kept under 5°C. The mixture was then heated and stirred at 75°C under reflux for 4 hours. The resulting reaction mixture was cooled to 10°C, and water (40 mL) was slowly added dropwise under ice bath cooling to produce a yellow solution. After washing with ethyl acetate, the aqueous phase (pH 2) was cooled to 5°C. Then, 40%

aqueous sodium hydroxide was added to adjust the pH to 3 to initiate crystallization. The resulting slurry was stirred at 3 to 6°C for 2 hours, kept in −18°C freezer for 2 hours, then filtered. The filter was rinsed with cold water (15 mL) and dried in vacuo (60°C, 5 hours) to produce (*S*)-**3** as a white solid (3.33 g, 56% yield). ^{1}H NMR (600 MHz, D_2O) δ 8.41 (s, 1H), 8.40 (s, 1H), 4.50 (dd, J = 14.8, 3.2 Hz, 1H), 4.31 (dd, J = 14.8, 7.0 Hz, 1H), 4.01 (pd, J = 6.4, 3.4 Hz, 1H), 3.70 (dd, J = 13.2, 9.2 Hz, 1H), 3.51 (dd, J = 13.2, 9.7 Hz, 1H), 1.19 (d, J = 6.3 Hz, 3H).

Phenyl Hydrogen ((((*S*)-1-(6-amino-9*H*-purin-9-yl) propan-2-yl)oxy)methyl)phosphonate ((*S*)-1)

To a stirring mixture of (*S*)-**3** (1.30 g, 4.5 mmol) and sieve-dried acetonitrile (12 mL) in a 50 mL three-necked round-bottomed flask under nitrogen was added sequentially triethylamine (0.92 g, 9.1 mmol), DMAP (0.55 g, 4.5 mmol), and triphenyl phosphite (2.11 g, 6.8 mmol). The reaction mixture was heated and stirred at 80°C for 48 hours when the reaction was judged complete by TLC (CH_2Cl_2:MeOH = 1:1, one drop of triethylamine). Then the solvents were evaporated under reduced pressure (60°C) to afford an oil residue, which was partitioned between ethyl acetate (10 mL) and water (25 mL). The aqueous layer was collected, washed with ethyl acetate (20 mL × 2), and then the pH was adjusted to 3 with 37% aqueous HCl to initiate crystallization. The resulting slurry was stirred at 30°C for 30 minutes. After the pH was further adjusted to 2 with 37% aqueous HCl, the slurry was stirred at 20°C for 30 minutes, 10°C for 2 hours, and then filtered. The filter was washed with cold pH 2 aqueous HCl (2.6 mL), and dried in vacuo (45°C, 4 hours) to afford (*S*)-**1** as a white solid (0.71 g, 43% yield, RP-HPLC purity: 98.43%). ^{1}H NMR (600 MHz, D_2O) δ 8.34 (s, 1H), 8.25 (s, 1H), 7.24 (t, J = 7.8 Hz, 2H), 7.11 (t, J = 7.4 Hz, 1H), 6.74 (d, J = 8.0 Hz, 2H), 4.44 (dd, J = 14.8, 2.6 Hz, 1H), 4.29 (dd, J = 14.8, 8.9 Hz, 1H), 4.13–4.05 (m, 1H), 3.85 (dd, J = 13.7, 7.8 Hz, 1H), 3.59 (dd, J = 13.7, 9.0 Hz, 1H), 1.31 (d, J = 6.2 Hz, 3H). mp: 224.8–227.4° C. $[\alpha]_{589}^{20}$ +4.8° (c = 1, 0.03 mol/L aqueous NaOH).

Diphenyl (*S*)-(((1-(6-amino-9*H*-purin-9-yl)propan-2-yl) oxy)methyl)phosphonate ((*S*)-2)

To a stirring mixture of (*S*)-**3** (0.7 g, 2.4 mmol) and sieve-dried acetonitrile (4 mL) in a 10 mL three-necked round-bottomed flask was added thionyl chloride (1.16 g, 9.7 mmol) at ambient temperature. The reaction mixture was heated and stirred under reflux for 3 hours. The resulting mixture was evaporated under reduced pressure to deliver a yellow oil residue, which was dispersed in sieve-dried acetonitrile (4 mL). The dispersion was cooled to –30°C, and phenol (0.5 g, 5.4 mmol) was added followed by dropwise addition of triethylamine to adjust the pH to 7–8. The resulting mixture was stirred at –30°C under nitrogen for 2.5 hours and then evaporated under reduced pressure to deliver a yellow solid residue, which was purified on a silica gel column (EtOAc: MeOH = 100:1→50:1) and then recrystallized from a mixture of ethyl acetate and *n*-heptane (1:1) to deliver (*S*)-**2** as a white solid (0.45 g, 42% yield, RP-HPLC purity: 99.32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (s, 1H), 8.04 (s, 1H), 7.41–7.32 (m, 4H), 7.26–7.18 (m, 4H), 7.13 (d, J = 8.2 Hz, 2H), 7.06 (d, J = 8.2 Hz, 2H), 4.33–4.14 (m, 4H), 4.11–4.03 (m, 1H), 1.13 (d, J = 6.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.96, 152.40, 149.79, 149.63 (d, J = 5.4 Hz), 149.55 (d, J = 6.0 Hz), 141.31, 129.92 (d, J = 4.2 Hz), 125.40, 125.36, 120.53 (d, J = 4.0 Hz), 120.46 (d, J = 4.0 Hz), 118.42, 75.73 (d, J = 13.0 Hz), 61.23 (d, J = 166.0 Hz), 46.83, 16.62. ^{31}P NMR (162 MHz, DMSO-d_6) δ 16.03. mp: 130.8–131.1°C.

Analytical Method Validation

HPLC analysis was performed on Dionex UltiMate 3000 System as specified in entry 4 (►Table 1). CSP: Daicel Chiralpak AD-H (4.6 mm × 250 mm, 5 µm), isocratic elution (A, *n*-hexane (0.1% diethylamine); B, isopropanol (0.1% diethylamine); B 50%) for 30 minutes, flow rate: 0.6 mL/min, detector: UV 260 nm, column temperature: 35°C, injection volume: 10 µL.

Sartorius CP225D analytical balance was used for weighing. *n*-Hexane, isopropanol, and water used are suitable for HPLC. Reference substances and samples were all obtained from Shanghai Institute of Pharmaceutical Industry Co., Ltd. Assays, *ee* values, and batch numbers of reference substances were as follows: (*R*)-**2** reference substance (98.30%, 100%, 220301), (*S*)-**2** reference substance (99.48%, 100%, 220303). Batch numbers of (*R*)-**2** samples for determination were 220301, 220915–1, and 220915–2.

Solution preparation: Blank solution: *n*-hexane: isopropanol, 1:1 (v/v). Reference stock solution of (*R*)-**2** or (*S*)-**2**: dissolve 10 mg of (*R*)-**2** or (*S*)-**2** reference substance in 10 mL of the blank solution, dilute 1 mL of the solution to 100 mL with the blank solution. Reference solution of (*R*)-**2** or (*S*)-**2**: dilute 1 mL of reference stock solution to 10 mL with the blank solution.

Specificity test: Dissolve 10 mg of (*R*)-**2** and 1 mL of reference stock solution of (*S*)-**2** in 10 mL of the blank solution to obtain the system suitability solution. HPLC measurements were performed for the system suitability solution and the blank solution. Specificity of this HPLC method proved to be good with the resolution between (*R*)-**2** and (*S*)-**2** being 5.70, and the blank solution not interfering with the determination of the sample.

Linearity: Dilute the reference stock solution of (*R*)-**2** with the blank solution to obtain serial w/v solutions of 0.2001, 0.5003, 1.0007, 1.5010, 2.0013, and 5.0034 µg/mL. Dilute the reference stock solution of (*S*)-**2** with the blank solution to obtain serial w/v solutions of 0.2021, 0.5054, 1.0107, 1.5161, 2.0214, and 5.0536 µg/mL. HPLC determinations were run for these solutions. A graph was plotted for linear regression with peak area (A) as the longitudinal coordinate and w/v concentration (c) as the horizontal coordinate. The regression equation for (*R*)-**2** is $A = 0.5405c + 0.0444$ ($r = 0.9997$) in the range of 0.2001 to 5.0034 µg/mL. The regression equation for (*S*)-**2** is $A = 0.5383c + 0.0224$ ($r = 0.9998$) in the range of 0.2021 to 5.0536 µg/mL.

Limit of detection (LOD) and limit of quantification (LOQ): LOD and LOQ values were calculated based on an S/N of 3 and 10, respectively. LOD and LOQ of (*R*)-**2** are 0.2001 and 0.5003 µg/mL, respectively. LOD and LOQ of (*S*)-**2** are 0.2021 and 0.5054 µg/mL, respectively.

Stability test: The system suitability solution was maintained at room temperature and sampled at 0, 3, 6, 9, 12, and 24 hours for HPLC analysis. The results indicated that the RSDs ($n = 6$) of retention time and peak area of (*R*)-**2** are 0.25 and 1.19%, respectively; the RSDs ($n = 6$) of retention time and peak area of (*S*)-**2** are 0.20 and 1.39%, respectively. Thus, the solution is stable for 24 hours at room temperature.

Precision test: HPLC analysis of the system suitability solution was run repeatedly six times. The results indicated that the RSDs ($n = 6$) of retention time and peak area of (*R*)-**2** are 0.04 and 0.54%, respectively; the RSDs ($n = 6$) of retention time and peak area of (*S*)-**2** is 0.11 and 0.98%, respectively.

Accuracy test: Accuracy was estimated by spiking 10 mg of (*R*)-**2** ($n = 9$) with 0.8, 1, and 1.2 mL of reference stock solution of (*S*)-**2**, then diluting to 10 mL with the blank solution to prepare 80, 100, and 120% recovery test solutions, respectively. HPLC analysis indicated that average recovery and RSD ($n = 3$) in the 80% group are 103.97 and 1.26%, those ($n = 3$) in the 100% group are 96.94 and 2.92%, and those ($n = 3$) in the 120% group are 98.78 and 4.90%, respectively.

Method robustness: The system suitability solution was tested by deliberate small variations of the HPLC conditions around the working conditions, i.e., flow rate: 0.6 ± 0.1 mL/min, column temperature: 35 ± 5°C, mobile phase solution B%: 50 ± 2%, respectively. The results indicated that the method is robust and not affected by minor changes in the HPLC parameters.

Sample determination: Test solution was prepared by dissolving 10 mg of (*R*)-**2** sample in 10 mL of the blank solution, and then determined by HPLC analysis. No (*S*)-**2** was detected in all three batches of (*R*)-**2** samples for determination.

Supporting Information

The ^{1}H NMR and ^{13}C NMR spectra of synthesized compounds as well as RP-HPLC traces of compounds (*R*)-**1**, (*R*)-**2**, (*S*)-**1**, and (*S*)-**2** are compiled in the **Supporting Information** (►**Figs. S1–S15** [online only]).

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Conflict of Interest
The authors declare no conflict of interest.

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