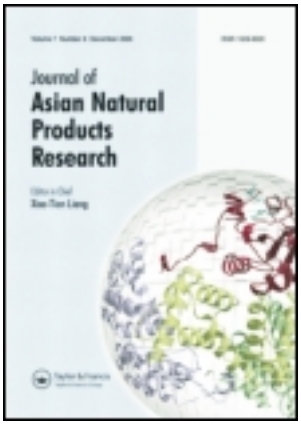

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Two new furostanol saponins from the seeds of *Trigonella foenum-graecum*

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Two new furostanol saponins, together with two known steroidal saponins, were isolated from the seeds of *Trigonella foenum-graecum* L. The structures of the new compounds were determined by detailed analysis of 1D NMR, 2D NMR, MS spectra and chemical evidences as 26-*O*-β-D-glucopyranosyl-(25*S*)-5-en-furost-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**1**) and 26-*O*-β-D-glucopyranosyl-(25*R*)-5-en-furost-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)]-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**2**).

Keywords: *Trigonella foenum-graecum*; Leguminosae; fenugreek seeds; furostanol saponins

1. Introduction

Trigonella foenum-graecum L. (fenugreek, Leguminosae), which is an annual herb, has been widely cultivated in China. The seeds of this plant have long been known as a traditional medicine used for tonic and stomachic purpose. The steroidal saponins, which are the principal constituents of fenugreek seeds, have some potential bioactivities [1–4]. Previous phytochemical studies had suggested that fenugreek seeds abounded in furostanol saponins, and many of them had been isolated [5–7]. Recently, our phytochemical investigation on fenugreek seeds led to the isolation of two new furostanol saponins, together with two known saponins. This paper mainly describes the separation and structure elucidation of these compounds.

2. Results and discussion

Phytochemical investigation was carried out on the extract of Chinese fenugreek seeds by column chromatography on macroporous resin SP825, silica-gel and ODS silica-gel, and semi-preparative RP-HPLC to yield two new compounds **1** and **2**, along with two known compounds **3** and **4** identified as trigoneoside Va and trigoneoside Vb, respectively, by comparison of their NMR spectral data with those in the literature [6] (Figure 1).

Compound **1**, obtained as a white amorphous powder, was deduced to be a furostanol saponin on the basis of the positive Ehrlich test. The IR spectrum showed the absorption bands at 3413, 2903, 1073, and 1050 cm^{-1}. The HR-ESI-MS provided an ion peak $[M - H]^-$ at *m/z* 1387.6411, which revealed its molecular

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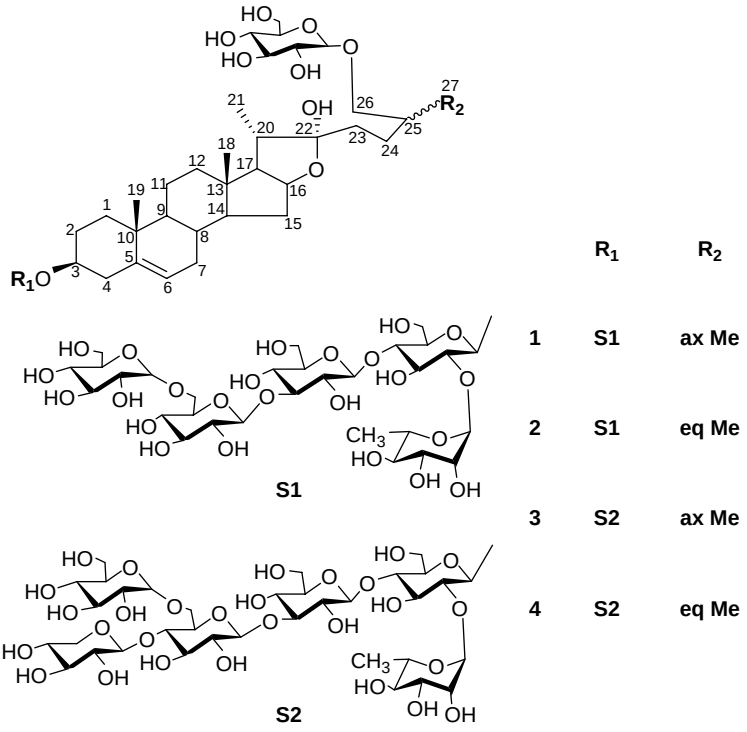

Figure 1. Structures of compounds **1**–**4**.

formula as $C_{63}H_{104}O_{33}$. The prominent fragments at *m/z* 1225 $[M - H\text{-}162]^-$, 1063 $[M - H\text{-}162 \times 2]^-$, 901 $[M - H\text{-}162 \times 3]^-$, 755 $[M - H\text{-}162 \times 3\text{-}146]^-$, 593 $[M - H\text{-}162 \times 4\text{-}146]^-$, and 431 $[M - H\text{-}162 \times 5\text{-}146]^-$ suggested the existence of five hexoses and one deoxyhexose units in its structure. By analysis of ^{1}H NMR, ^{13}C NMR and HSQC spectra, six anomeric proton signals at δ 4.92 (1H, d, $J = 9.0$ Hz, H-1′), 6.21 (1H, br s, H-1″), 5.06 (1H, d, $J = 7.2$ Hz, H-1‴), 5.16 (1H, d, $J = 7.2$ Hz, H-1⁗), 4.94 (1H, d, $J = 7.2$ Hz, H-1⁗′), 4.80 (1H, d, $J = 7.8$ Hz, H-1⁗″), and six corresponding anomeric carbon signals at δ 100.0 (C-1′), 101.8 (C-1″), 104.6 (C-1‴), 105.5 (C-1⁗), 104.8 (C-1⁗′) and 105.2 (C-1⁗″) were assigned, which also suggested the presence of six sugar units. Acid hydrolysis led to the identification of D-glucoses and L-rhamnose. Compound **1** had the same aglycone carbon signals as trigoneoside Va due to their almost identical ^{13}C NMR spectral data, and all proton signals for the aglycone moiety of **1** were assigned according to ^{1}H–^{1}H COSY and HSQC experiments. In addition, all proton and carbon signals of sugar moieties were identified based on the combined use of ^{1}H–^{1}H COSY, HSQC, and HMBC spectra. β-configurations of five D-glucoses were determined by the $J_{1,2}$ values (>7.0 Hz): 9.0, 7.2, 7.2, 7.2, and 7.8 Hz, respectively [8]. The chemical shifts of C-3″ at δ 72.8 and C-5″ at δ 69.5 indicated the α-anomeric configuration of L-rhamnose [9]. The C-25 configuration of **1** was deduced to be *S* due to the chemical shift difference of two protons H_a-26 at δ 4.07 and H_b-26 at 3.48 ($\Delta ab = 0.59 > 0.57$) [10]. The α-configuration of OH-22 was deduced from the hemiketal carbon signal at δ 110.7, about 2 ppm higher than that of the β-configuration [11,12]. In the HMBC spectrum, the key correlations between

H-1$''''''$ at δ 4.80 and C-26, H-1$'$ at δ 4.92 and C-3 indicated the glycosylation positions of sugar units, respectively. Meanwhile, the correlations between H-1$''$ at δ 6.21 and C-2$'$ at δ 77.3, H-1$'''$ at δ 5.06 and C-4$'$ at δ 81.4, H-1$''''$ at δ 5.16 and C-3$'''$ at δ 88.5, H-1$'''''$ at δ 4.94 and C-6$''''$ at δ 70.1 decided the sequence of sugar chain at C-3 (Figure 2). Consequently, the structure of **1** was elucidated to be 26-*O*-β-D-glucopyranosyl-(25*S*)-5-en-furost-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside.

Compound **2** was isolated as a white amorphous powder giving the positive Ehrlich test. The IR spectrum showed the absorption bands at 3412, 2904, 1073, and 1050 cm^{-1}. The ion $[M - H]^-$ at *m/z* 1387.6409 in HR-ESI-MS indicated the molecular formula of **2** to be $C_{63}H_{104}O_{33}$. ESI-MS experiment showed the prominent fragment ion peaks at *m/z* 1225 $[M - H\text{-}162]^-$, 1063 $[M - H\text{-}162 \times 2]^-$, 901 $[M - H\text{-}162 \times 3]^-$, 755 $[M - H\text{-}162 \times 3\text{-}146]^-$, 593 $[M - H\text{-}162 \times 4\text{-}146]^-$, and 431 $[M - H\text{-}162 \times 5\text{-}146]^-$. The correlations between six anomeric proton signals at δ 4.93 (1H, d, $J = 9.0$ Hz, H-1$'$), 6.20 (1H, br s, H-1$''$), 5.05 (1H, d, $J = 7.2$ Hz, H-1$'''$), 5.16 (1H, d, $J = 7.2$ Hz, H-1$''''$), 4.94 (1H, d, $J = 7.2$ Hz, H-1$'''''$), 4.80 (1H, d, $J = 7.8$ Hz, H-1$''''''$) and six corresponding carbon signals at δ 100.0 (C-1$'$), 101.8 (C-1$''$), 104.6 (C-1$'''$), 105.5 (C-1$''''$), 104.8 (C-1$'''''$), 105.0 (C-1$''''''$) were observed in HSQC spectrum. D-glucoses and L-rhamnose were detected by acid hydrolysis. The above evidence confirmed that **2** was a furostanol saponin with five D-glucoses and one L-rhamnose units. Compound **2** had the same molecular formula as **1** and its spectral data were almost identical to those of **1**, which suggested that **2** was one epimer of **1**. The proton signals of **2** were assigned according to the ^1H–^1H COSY and HSQC experiments. The chemical shifts of H_a-26 at δ 3.94 and H_b-26 at δ 3.62 ($\Delta ab = 0.32 < 0.48$), which are different from those of **1**, indicated the 25*R* configuration of **2**. Therefore, the structure of **2** was determined as 26-*O*-β-D-glucopyranosyl-(25*R*)-5-en-furost-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with PerkinElmer 343 polarimeter (PerkinElmer,

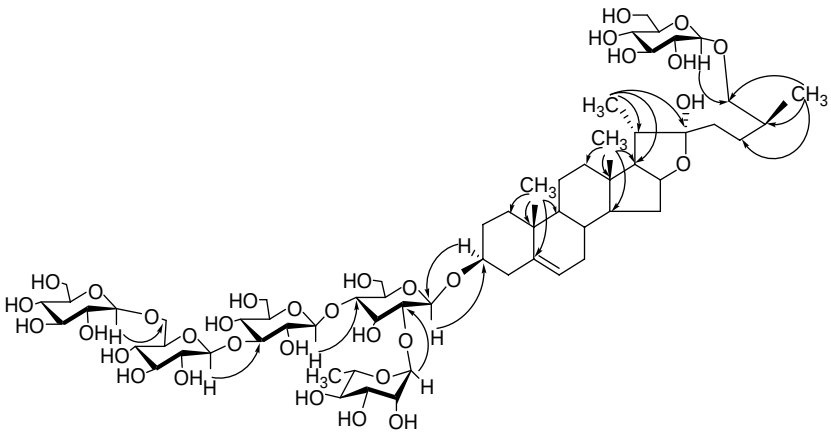

Figure 2. The key HMBC correlations of compound **1**.

Waltham, MA, USA). The measurements of IR were taken using Bruker Vertex 70 (Bruker Corporation, Karlsruhe, Germany). Gas chromatographic analysis was performed with an Agilent 6890 system (Agilent Technologies Co. Ltd, Santa Clara, CA, USA) equipped with an H_2 flame ionization detector. The column was a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm). The NMR spectra were recorded with Varian^{UNITY} INOVA 600 (599.8 MHz for ^{1}H NMR and 150.8 MHz for ^{13}C NMR) (Palo Alto, CA, USA) and the chemical shifts were given in δ (ppm) scale with tetramethylsilane as an internal standard. The HR-ESI-MS was recorded on Synapt Q-Tof MS (Waters Corporation, Milford, MA, USA). HPLC was performed using Waters 2695 system (Waters Corporation), Alltech 2000 Evaporative Light Scattering Detector (Alltech, Lexington, KY, USA), Megres C18 (4.6 × 250 mm, ODS, 5 μm) (Hanbon Sci. Tech., Huaian, China). HPLC separations were performed using Primeline solvent delivery module (ASI, Salt Lake City, UT, USA) equipped with a Shodex RID 102 detector (Showa Denko Group, Tokyo, Japan) and a Dubhe C18 column (8.0 × 250 mm, ODS, 5 μm) (Hanbon Sci. Tech.). TLC was performed on precoated silica gel GF254 plates (Qingdao Marine Chemical Co. Ltd., Qingdao, China). Macroporous resin SP825 (Mitsubishi Chemical, Tokyo, Japan), silica-gel H (Qingdao Marine Chemical Co. Ltd), and ODS silica-gel (120 Å, 50 μm, YMC, Kyoto, Japan) were used for column chromatography.

3.2 Plant material

The seeds of *T. foenum-graecum* were purchased from Anguo, Hebei province, China in June 2008, and were identified by Prof. Li-juan Zhang (Tianjin University of Traditional Chinese Medicine). A voucher specimen has been deposited in our laboratory with the number 20080618.

3.3 Extraction and isolation

Air-dried and powdered fenugreek seeds (4.5 kg) were extracted three times with hot 70% EtOH–H_2O, which was carried out for 1 h each time. The combined extract was filtered and concentrated under reduced pressure. The residue was subjected to macroporous resin SP825 column chromatography with a gradient mixture of EtOH–H_2O (15:85, 55:45, and 95:5) to provide fraction A, fraction B (296 g), and fraction C (22 g). Fraction B was further fractionated by macroporous resin SP825 using EtOH–H_2O (25:75, 35:65, 45:55, and 95:5) to give four fractions B1 (22 g), B2 (12 g), B3 (121 g), and B4 (71 g). A part of fraction B3 (9 g) was chromatographed on ODS C_{18} column (5 × 40 cm) with Me_2CO–H_2O (22:78 → 25:75) to yield seven important combined fractions: Fr. 33-40 (558 mg), Fr. 41-48 (300 mg), Fr. 49-55 (330 mg), Fr. 56-70 (370 mg), Fr. 98-108 (236 mg), Fr. 115-173 (2680 mg), and Fr. 179-240 (1980 mg). Fr. 115-173 (2000 mg) was chromatographed on silica-gel column (3.5 × 30 cm) with $CHCl_3$–MeOH–H_2O (65:20:2 → 65:35:10), and a total of 88 tubes (100 ml each) were collected. Of these tubes, tubes 59-60 (164 mg) were further purified repeatedly by RP-HPLC with a CH_3CN–H_2O (24:76) mobile phase to give compounds **1** (23.6 mg) and **2** (33.0 mg). Tubes 75–87 (300 mg) were chromatographed by RP-HPLC using CH_3CN–H_2O (24:76) to give compounds **3** (45.6 mg) and **4** (76.7 mg).

3.3.1 Compound *1*

White amorphous powder, $C_{63}H_{104}O_{33}$, $[\alpha]_D^{20}$ −61 (c = 0.07, pyridine). IR (KBr, cm^{-1}): 3413, 2903, 1159, 1073, 1050. ^{1}H NMR (pyridine-d_5, 599.8 MHz) and ^{13}C NMR (pyridine-d_5, 150.8 MHz) spectral data see Tables 1 and 2. ESI-MS (negative mode) *m/z*: 1225 [M − H-162]$^-$, 1063 [M − H-162 × 2]$^-$, 901 [M − H-162 × 3]$^-$, 755 [M − H-162 × 3-146]$^-$, 593

Table 1. ^{1}H and ^{13}C NMR spectral data of the aglycone moiety of **1** and **2**.

	1		**2**	
No.	^{13}C	^{1}H	^{13}C	^{1}H
1	37.5	1.73 m, 0.97 m	37.5	1.74 m, 0.98 m
2	30.2	2.09 m, 1.86 m	30.2	2.09 m, 1.86 m
3	78.1	3.88 m	78.1	3.90 m
4	39.0	2.75 m, 2.70 m	39.0	2.74 m, 2.70 m
5	140.8		140.8	
6	121.9	5.23 m	121.9	5.28 m
7	32.4	1.87 m, 1.48 m	32.4	1.86 m, 1.49 m
8	31.7	1.56 m	31.7	1.56 m
9	50.4	0.89 m	50.4	0.90 m
10	37.2		37.2	
11	21.1	1.46 m, 1.42 m	21.1	1.45 m, 1.42 m
12	40.0	1.73 m, 1.11 m	40.0	1.74 m, 1.13 m
13	40.8		40.8	
14	56.6	1.07 m	56.6	1.07 m
15	32.5	2.02 m, 1.46 m	32.5	2.02 m, 1.48 m
16	81.1	4.94 m	81.1	4.94 m
17	63.9	1.92 m	63.9	1.93 m
18	16.5	0.91 s	16.5	0.92 s
19	19.4	1.04 s	19.4	1.06 s
20	40.7	2.22 m	40.7	2.22 m
21	16.5	1.31 d (7.2)	16.5	1.31 d (7.2)
22	110.7		110.7	
23	37.2	2.07 m, 1.98 m	37.2	2.04 m, 2.00 m
24	28.4	2.06 m, 1.68 m	28.4	2.05 m, 1.69 m
25	34.5	1.92 m	34.3	1.92 m
26	75.3	4.07 m, 3.48 dd (6.0, 9.0)	75.2	3.94 m, 3.62 dd (6.0, 9.0)
27	17.5	1.02 d (6.6)	17.5	0.99 d (6.6)

Note: ^{1}H and ^{13}C NMR signals were assigned on the basis of ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

$[M - H\text{-}162 \times 4\text{-}146]^-$, 431 $[M - H\text{-}162 \times 5\text{-}146]^-$. HR-ESI-MS (negative mode): *m/z* 1387.6411 $[M - H]^-$ (calcd for $C_{63}H_{103}O_{33}$, 1387.6382).

3.3.2 *Compound* **2**

White amorphous powder, $C_{63}H_{104}O_{33}$, $[\alpha]_D^{20}$ −49 ($c = 0.066$, pyridine). IR (KBr, cm^{-1}): 3412, 2904, 1159, 1073, 1050. ^{1}H NMR (pyridine-d_5, 599.8 MHz) and ^{13}C NMR (pyridine-d_5, 150.8 MHz) spectral data see Tables 1 and 2. ESI-MS (negative mode) *m/z*: 1225 $[M - H\text{-}162]^-$, 1063 $[M - H\text{-}162 \times 2]^-$, 901 $[M - H\text{-}162 \times 3]^-$, 755 $[M - H\text{-}162 \times 3\text{-}146]^-$, 593 $[M - H\text{-}162 \times 4\text{-}146]^-$, 431 $[M - H\text{-}162 \times 5\text{-}146]^-$, 413 $[M - H\text{-}162 \times 5\text{-}146\text{-}18]^-$. HR-ESI-MS (negative mode): *m/z* 1387.6409 $[M - H]^-$ (calcd for $C_{63}H_{103}O_{33}$, 1387.6382).

3.4 *Acid hydrolysis*

A mixture of **1** and **2** (15 mg) was dissolved in 2 N aq. CF_3COOH (5 ml) and heated for 4 h at 95°C. After extracting with CH_2Cl_2 (5 ml) for three times, the water layer was repeatedly evaporated to dryness with EtOH to get rid of CF_3COOH. Then, in monosaccharide mixture, glucose and rhamnose were detected by comparing with authentic samples over TLC ($CHCl_3$–MeOH–H_2O 8:5:1): glucose (R_f 0.46) and rhamnose (R_f 0.60). Furthermore, the residue of sugars was dissolved in anhydrous pyridine (2 ml), and L-cysteine methyl ester hydrochloride (12 mg) was added. The obtained mixture

Table 2. ^{1}H and ^{13}C NMR spectral data of the sugar moieties of **1** and **2**.

	1		**2**	
No.	^{13}C	^{1}H	^{13}C	^{1}H
3-Glc-1′	100.0	4.92 d (9.0)	100.0	4.93 d (9.0)
2′	77.3	4.20 m	77.2	4.19 m
3′	76.3	3.80 m	76.3	3.81 m
4′	81.4	4.18 m	81.5	4.18 m
5′	77.6	3.86 m	77.6	3.86 m
6′	61.6	4.34 m, 4.24 m	61.6	4.34 m, 4.22 m
2′-Rha-1″	101.8	6.21 br s	101.8	6.20 br s
2″	72.5	4.73 m	72.5	4.72 m
3″	72.8	4.58 m	72.8	4.58 m
4″	74.2	4.34 m	74.2	4.33 m
5″	69.5	4.92 m	69.5	4.92 m
6″	18.7	1.76 d (6.0)	18.7	1.76 d (6.0)
4′-Glc-1‴	104.6	5.06 d (7.2)	104.6	5.05 d (7.2)
2‴	73.4	4.02 m	73.4	4.03 m
3‴	88.5	4.12 m	88.5	4.12 m
4‴	69.4	4.16 m	69.4	4.17 m
5‴	77.6	4.18 m	77.7	4.18 m
6‴	61.5	4.49 m, 4.44 m	61.5	4.47 m, 4.40 m
3‴-Glc-1⁗	105.5	5.16 d (7.2)	105.5	5.16 d (7.2)
2⁗	75.1	4.01 m	75.1	4.02 m
3⁗	76.9	4.15 m	76.9	4.15 m
4⁗	71.9	3.99 m	71.9	4.01 m
5⁗	75.4	4.07 m	75.3	4.05 m
6⁗	70.1	4.90 m, 4.06 m	70.1	4.90 m, 4.04 m
6⁗-Glc-1⁗′	104.8	4.94 d (7.2)	104.8	4.94 d (7.2)
2⁗′	75.6	4.06 m	75.6	4.06 m
3⁗′	78.3	3.90 m	78.3	3.90 m
4⁗′	71.4	4.24 m	71.4	4.23 m
5⁗′	78.5	4.25 m	78.5	4.24 m
6⁗′	62.5	4.45 m, 4.35 m	62.5	4.46 m, 4.36 m
26-Glc-1⁗″	105.2	4.80 d (7.8)	105.0	4.80 d (7.8)
2⁗″	75.3	4.02 m	75.2	4.02 m
3⁗″	78.6	4.23 m	78.6	4.23 m
4⁗″	71.7	4.24 m	71.7	4.21 m
5⁗″	78.5	3.94 m	78.5	3.94 m
6⁗″	62.9	4.54 m, 4.38 m	62.9	4.52 m, 4.36 m

Note: ^{1}H and ^{13}C NMR signals were assigned on the basis of ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

was then stirred at 60°C for 1 h. After that, hexamethyldisilazane–trimethylchlorosilane; 2:1 (6 ml) was added, and the mixture was further kept at 60°C for 0.5 h [13]. Finally, the supernatant was analyzed by GC: Agilent Technologies 6890 gas chromatograph equipment; H_2 flame ionization detector; HP-5 capillary column (30 m × 0.25 mm × 0.25 μm); column temperature: 180°C–250°C, programmed increase, 15°C/min; carrier gas: N_2 (1 ml/min); injection and detector temperature: 250°C; injection volume: 1.0 μl; split ratio: 1/50. Consequently, the D-configuration of glucose and the L-configuration of rhamnose were established by comparing their retention times with those of standard samples, respectively. t_R: D-glucose (17.91, 19.67 min) and L-rhamnose (14.48, 16.24 min).

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