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Three new steroidal saponins from *Helleborus thibetanus*

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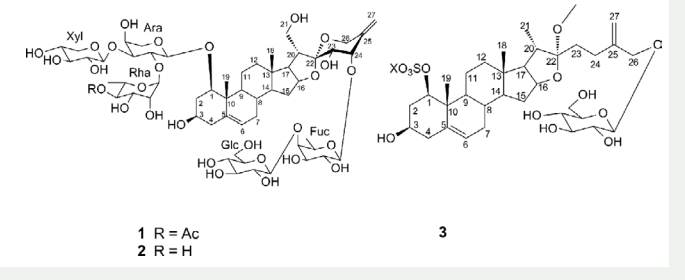
ABSTRACT

Three new steroidal saponins including two spirostanol glycosides (**1–2**) and one furostanol glycoside 1-sulphate (**3**) were isolated from the dried roots and rhizomes of *Helleborus thibetanus*. Structures of the compounds were determined on the basis of extensive use of 1-D and 2-D NMR experiments, together with HR–ESI–MS and IR measurements, as well as the results of acid hydrolysis. Compounds **1–2** represented steroidal saponins with an unusual substitution pattern, which possessed a double bond at C-25 and were glycosylated at 1-OH.



1 R = Ac
2 R = H

3

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Helleborus; Ranunculaceae; steroidal saponins; spirostanol; furostanol

1. Introduction

Helleborus, a genus of herbaceous perennials belongs to the family Ranunculaceae. A total of around 25 species are distributed over different parts of South-east Europe and West Asia (Sylla et al. 2014). It is reported that steroids including bufadienolides, phytoecdystones and steroidal saponins (Cheng et al. 2014; Zhang et al. 2014a, 2014b) constitute the major components of *Helleborus* according to previous phytochemical research. Some *Helleborus* species extracts possess immunostimulatory, anti-inflammatory and different anticancer properties (Čakar et al. 2014). *Helleborus thibetanus* (FRANCH), a plant endemic to China, is mainly growing wild in Sichuan, Gansu and Shaanxi. The roots and rhizomes of *H. thibetanus*, commonly known by the local name of 'Xiao-Tao-Er-Qi', have been used for the treatment of traumatic injury, cystitis and urethritis. Several steroidal saponins, one pregnane, one

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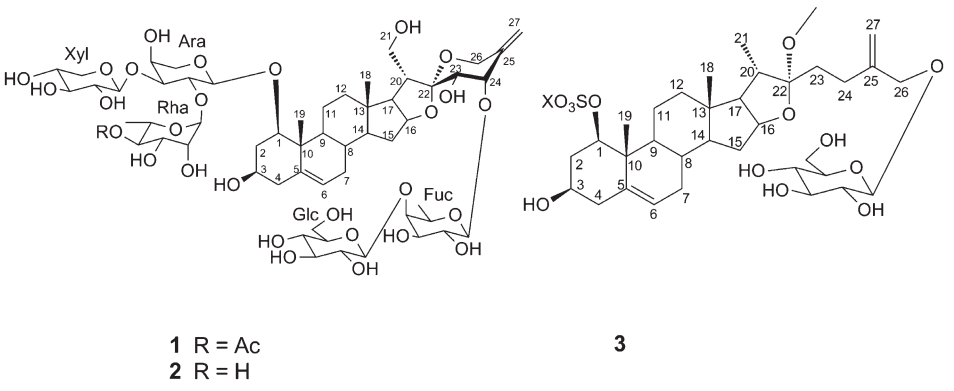

Figure 1. The structures of compounds **1–3**, **3** given as salt (mostly K^+).

spirostanol sulphate, several bufadienolides and phytoecdystones had been isolated from *H. thibetanus* (Zhang et al. 2014a, 2014b). On continuing the study of this plant, we have now isolated two new spirostanol steroidal saponins (**1–2**) and one new furostanol glycoside 1-sulphate (**3**) (Figure 1) from the title plant. This paper deals with the isolation and structural elucidation of the three new steroidal saponins by detailed analysis of their NMR spectra and acid hydrolysis.

2. Results and discussion

Compound **1** was obtained as a white amorphous solid and its molecular formula was assigned as $C_{57}H_{88}O_{29}$, deduced from the HR–ESI–MS *m/z* 1235.5327 ($[M - H]^-$), as well as its ^1H and ^{13}C NMR spectroscopic data. The IR spectrum of **1** showed the characteristic absorptions of hydroxyl groups at 3396 cm^{-1} and carbonyl group at 1729 cm^{-1}. Its ^1H and ^{13}C NMR spectra revealed the presence of two angular Me groups at δ_H 1.02 (3H, s), 1.36 (3H, s) and δ_C 16.7, 14.9, and a characteristic quaternary carbon signal at δ_C 111.7, suggesting the occurrence of a spirostanol skeleton in **1**. The position of 21-OH was established based on the HMBC correlations between the protons at δ_H 1.93 (1H, m, H-17) and δ_H 3.31 (1H, m, H-20) and the carbon resonance at δ_C 62.2 (C-21). The correlation from the olefinic proton at δ_H 5.62 (1H, br d, J = 5.0 Hz) to δ_C 124.7 (C-6) was observed in the HSQC spectrum, identifying the double bond at C-5(6), which was also verified by the correlations from olefinic proton at δ_H 5.62 (1H, br d, J = 5.0 Hz) to the carbon resonances of δ_C 43.8 (C-4), δ_C 33.0 (C-8) and δ_C 42.8 (C-10), along with correlations between δ_H 1.36 (3H, s, Me-19) and δ_C 139.4 (C-5) in the HMBC spectrum. Evidence for the presence of the other double bond at C-25(27) came from correlations from the olefinic protons at δ_H 5.16 (1H, m) and δ_H 5.04 (1H, br s) to δ_C 113.7 (C-27) in the HSQC spectrum, which was demonstrated by HMBC correlations from olefinic protons at δ_H 5.16 (1H, m) and δ_H 5.04 (1H, br s) to the carbon resonances of δ_C 82.2 (C-24), δ_C 143.7 (C-25) and δ_C 61.4 (C-26). The proton signal at δ_H 3.79 (1H, m) was assigned as H-1 attached to the oxygenated C-1 by its correlation with C-1 (δ_C 83.7) in the HSQC spectrum, which was also confirmed by the HMBC correlation between Me-19 (3H, s, δ_H 1.36) and C-1 (δ_C 83.7). The signal at δ_H 3.87 (1H, m) in the ^1H NMR spectrum showed COSY correlations with H-4ax/H-2ax, giving evidence for its assignment of H-3. In addition, the NOESY cross-peaks between H-1 and H-3, between Me-19 and Me-18/H-2ax/H-4ax, indicated

the β-configurations of the oxygenated substituents at C-1 and C-3. Moreover, the configurations of C-23 and C-24 were characterised as *S* by the NOESY correlations between H-23 and H-20, between H-23 and H_{2-21}/H_{2-27}, between H-24 and H_{2-27} (Mimaki & Watanabe 2008; Hayes et al. 2009). Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of the aglycone moiety of **1** with those of bethoside A (Hayes et al. 2009), along with the above analysis, the structure of the aglycone of **1** was elucidated as (23*S*,24*S*)-1β,3β,21,23,24-pentahydroxy-spirosta-5,25(27)-diene. For the sugar moiety, the five anomeric protons at δ_H 6.46 (1H, br s), 5.14 (1H, d, J = 7.0 Hz), 5.13 (1H, d, J = 7.0 Hz), 4.91 (1H, d, J = 7.0 Hz) and 4.67 (1H, d, J = 7.5 Hz) showed correlations with the anomeric carbon resonances at δ_C 100.7, 105.9, 106.8, 106.6 and 100.5 in the HSQC spectrum, respectively. And two Me groups were observed at δ_H 1.36 (3H, d, J = 5.5 Hz), 1.50 (3H, d, J = 6.0 Hz) in the ^{1}H NMR spectrum, and δ_C 18.3, δ_C 17.4 in the ^{13}C NMR spectrum, respectively, which implied two of them were 6-deoxyhexose units. The Me group at δ_H 2.00 (3H, s) and δ_C 20.9 and C=O signal at δ_C 170.7 were assignable to one acetyl group. Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1:1) followed by TLC analysis showed the presence of arabinose (Ara), rhamnose (Rha), xylose (Xyl), fucose (Fuc) and glucose (Glc). One glycosyl group attached to C-1 position of the aglycone was established from the HMBC correlations of signals at δ_H 4.67 (H-1 of Ara) with δ_C 83.7 (C-1 of the aglycone), H-1 (δ_H 6.46) of Rha with C-2 (δ_C 72.6) of Ara, H-1 (δ_H 4.91) of Xyl with C-3 (δ_C 85.0) of Ara, which was also supported by the NOESY correlations of signals at H-1 (δ_H 3.79) of aglycone with H-1 (δ_H 4.67) of Ara, H-2 (δ_H 4.59) of Ara with H-1 (δ_H 6.46) of Rha, H-3 (δ_H 4.07) of Ara with H-1 (δ_H 4.91) of Xyl. The other sugar chain located at C-24 (δ_C 82.2) of **1** was deduced by a downfield shift of 8.1 ppm of C-24 (δ_C 74.1) compared with clintonioside B (Mimaki & Watanabe 2008), and the HMBC correlations between H-24 (δ_H 4.75) of the aglycone and C-1 (δ_C 105.9) of Fuc, and between C-4 (δ_C 83.2) of Fuc to H-1 (δ_H 5.13) of Glc proved the linkage of the sugars and the aglycone, which was further supported by the NOESY cross-peaks between H-24 (δ_H 4.75) of aglycone and H-1 (δ_H 5.14) of Fuc, between H-4 (δ_H 4.04) of Fuc and H-1 (δ_H 5.13) of Glc. Full assignments of **1** were achieved by a comprehensive analysis of DEPT, COSY, HSQC, NOESY and HMBC spectra. On the basis of the above evidence, the structure of the new spirostanol glycoside **1** was fully determined to be (23*S*,24*S*)-21-hydroxymethyl-24-{[*O*-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosyl]oxy}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl *O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Compound **2**, a white amorphous solid, exhibited the ion peak at *m/z* 1193.5226 $[M - H]^-$ in the HRESIMS data, corresponding to the molecular formula $C_{55}H_{85}O_{28}$, which displayed the lack of C_2H_3O compared with **1**. The IR absorption at 3424 cm^{-1} revealed the presence of hydroxyl groups. Complete assignments of the ^{1}H and ^{13}C NMR signals of **2** were accomplished by a combined analysis of DEPT, COSY, HSQC, NOESY and HMBC spectra. A detailed comparison of the ^{1}H, ^{13}C NMR chemical shifts of **1** and **2**, revealed that they shared the same skeleton and same glycosidic positions at C-1 and C-24, except for the disappearance of the carbon signal at δ_C 170.7 and δ_C 20.9 in the ^{13}C NMR spectrum and the proton signal of δ_H 2.00 (3H, s) in the ^{1}H NMR spectrum of compound **2**. Meanwhile, the chemical shift of Me signal of Rha at δ_H 1.36 (3H, d, J = 5.5 Hz) moved downfield to δ_H 1.66 (3H, d, J = 6.0 Hz), these data suggested the acetyl linked at C-4 of Rha was absent in compound **2**. Acid hydrolysis of **2** with 1 M HCl in dioxane-H_2O (1:1) gave Ara, Rha, Xyl, Fuc and Glc. Thus, **2** was deduced to be (23*S*,24*S*)-21-hydroxymethyl-24-{[*O*-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosyl]oxy}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl *O*-(α-L-rhamnopy-ranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Compound **3** was isolated as a white amorphous solid. Its molecular formula was determined as $C_{34}H_{53}O_{13}S$, deduced from the HR–ESI–MS (*m/z* 701.3224 $[M]^-$), as well as its ^{13}C NMR spectrum. The ^1H NMR spectrum of **3** displayed signals for two tertiary methyl groups at δ_H 0.77 (3H, s) and 1.21 (3H, s), one secondary methyl groups at δ_H 1.04 (3H, d, J = 7.5 Hz), one methoxyl group at δ_H 3.18 (3H, s), one methine proton signal at 3.84 (1H, m) attributed to secondary alcoholic function, two methylene proton signals at δ_H 4.53 (1H, br d, J = 12.5 Hz) and 4.27 (1H, m), indicative of a primary alcoholic function, three olefinic protons at δ_H 5.53 (1H, br d, J = 5.5 Hz), δ_H 5.27 (1H, br s), δ_H 4.98 (1H, br s), along with an anomeric proton at δ_H 4.82 (1H, d, J = 7.5 Hz). Besides, the ^{13}C NMR spectrum for the aglycone moiety exhibited signals ascribable to an acetal carbon at δ_C 112.3, one secondary alcoholic function at δ_C 67.7, one primary alcoholic function at δ_C 71.9 and one methoxyl carbon at δ_C 47.2, together with an anomeric carbon at δ_C 103.7. The evidence above manifested that **3** had a glycosidic furostanol skeleton with one methoxy. The signal at δ_C 85.1 (C-1) of compound **3** was similar to δ_C 85.5 (C-1) of ruscogenin 1-sulphate (Asano et al. 1993), δ_C 84.1 (C-1) of spirost-5-en-1β,3β-diol 1-sulphate (Oulad-Ali et al. 1996) and δ_C 85.1 (C-1) of spirost-5,25(27)-dien-1β,3β-diol 1-sulphate (Yang et al. 2010), indicating a sulphate group at C-1. The existence of the sulphate functional group was further affirmed by a series of characteristic strong absorption bands at 1242, 1067 and 951 cm^{-1} in its IR (KBr) spectrum. The HMBC spectrum of **3** provided interaction of C-22 at δ_C 112.3 with the proton at δ_H 3.18 (3H, s), testifying that the methoxyl group connected to C-22. The occurrence of a double bond at C-25(27) was confirmed from a correlation signal between the proton signals at δ_H 5.27 (1H, br s, H-27a), δ_H 4.98 (1H, br s, H-27b) and the carbon signals of C-24 (δ_C 28.0) and C-26 (δ_C 71.9) in the HMBC spectrum. The assignments of all the individual protons and carbons of **3** were achieved with the analysis of COSY, HSQC, HMBC and NOESY spectra. In addition, the presence of a glucopyranosyl moiety in **3** was readily recognised by the appearance of an anomeric proton signal at δ_H 4.82 (1H, dd, J = 7.5 Hz) in the ^1H NMR spectrum and also by the characteristic six signals at δ_C 103.7, 74.9, 78.4, 71.6, 78.3 and 62.7 in the ^{13}C NMR spectrum. HMBC correlation of anomeric proton signal at δ_H 4.82 with C-26 (δ_C 71.9) proved the location of the glucopyranosyl moiety at C-26 of aglycone, which is a structural feature in naturally occurring furostanol glycosides (Matsuo et al. 2008). Acid hydrolysis of **3** with 1 M HCl in dioxane-H_2O (1:1), followed by TLC analysis indicated the presence of Glc. Eventually, the structure of **3** was unequivocally identified to be 26-*O*-β-D-glucopyranosyl-3β-hydroxy-22α-methoxyfurosta-5,25(27)-diene-1β-yl sulphate.

3. Experimental

3.1. General experimental procedures

Column chromatographic isolations were performed using silica gel (100–200 and 200–300 mesh, Qingdao Haiyang Chemical Co. Ltd, Qingdao, China), D101 macroporous resin (Tianjin Haiguang Chemical Co. Ltd, Tianjin, China), Sephadex LH–20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and ODS (40–63 μm, LiChroprep RP-18, Merck KGaA, Darmastadt, Germany), ODS was also used as a stationary phase for the medium-pressure liquid chromatography (MPLC) system. Analytical HPLC was carried out using ODS columns (Agilent ZORBAX SB–C18, 4.6 mm × 250 mm, 5 μm, CA, USA). Preparative HPLC was performed using ODS columns (Agilent ZORBAX SB–C18, 21.2 mm × 250 mm, 7 μm, CA, USA). Thin-layer

chromatography was conducted on precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co. Ltd, Qingdao, China), and spot detection was performed under fluorescent light and then spraying 5% H_2SO_4 in EtOH, followed by heating. NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Bruker Daltonics Inc., Switzerland) and a Varian INOVA 500 MHz spectrometer (Varian, CA, USA). High-resolution electrospray ionisation mass spectrometry (HR–ESI–MS) was obtained on a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics Inc., Germany). Optical rotations were measured on a Rudolph Research Analytical Autopol II automatic polarimeter (Rudolph Research Analytical, USA). IR spectra were determined on a Bruker Tensor 27 spectrometer (Bruker Co. Ettlingen, Germany) with KBr pellets. UV spectra were measured using an Agilent Cary 60 UV–vis spectrophotometer (Agilent, CA, USA). All solvents used were of analytical grade (Tianjin Jiangtian Chemical Technology Co. Ltd, Tianjin, China).

3.2. Plant material

The roots and rhizomes of *H. thibetanus* were collected in September of 2007 from Mei County, Shaanxi Province in the People's Republic of China and were authenticated by Prof. Zhen-Hai Wu, College of Life Sciences, Northwest A&F University, China. A voucher specimen (S200609002) has been deposited in School of Pharmaceutical Science and Technology, Tianjin University, Tianjin.

3.3. Extraction and isolation

The roots and rhizomes of *H. thibetanus* (8.0 kg dry weight) were air-dried. They were crushed and refluxed for 2 h with 6 L of 95% ethanol twice and then with 6 L of 60% ethanol once. The combined extracts were concentrated under reduced pressure to give a residue (1.5 kg) which was suspended in water to a final volume of 5 L and then exhaustively extracted at room temperature with the following solvents: petroleum ether (PE), $CHCl_3$, EtOAc and *n*-BuOH, thus yielding four extracts. The respective amount of the PE extract, $CHCl_3$ extract, EtOAc extract and *n*-BuOH extract was 46, 28, 48 and 934 g. The *n*-BuOH extract (934 g) was loaded onto a D101 macroporous resin column, and successively eluted with EtOH–H_2O (0:100, 30:70, 50:50, 70:30 and 95:5) to get 5 fractions. The fraction A (378 g), eluted with 30% EtOH, was submitted to a silica gel column using the eluent of EtOAc–MeOH (9:1→6:4) to provide 126 fractions. Fractions A56–A76 (54 g) were chromatographed over silica gel using EtOAc–MeOH mixtures of increasing polarity successively, producing fractions 29–58, repeatedly submitted to purification by silica gel column eluted with CH_2Cl_2–MeOH (84:16→0:100) and then subjected to Sephadex LH–20 chromatography using MeOH to yield compound **3** (30 mg). With a gradient of EtOAc–MeOH (80:20→0:100), fractions A93–A107 (68 g) were applied to a silica gel column to give 96 fractions. Fractions 63–78 were further chromatographed using MPLC over ODS with a step gradient of MeOH–H_2O (33:67→0:100). Combined subfractions 14–38 were rechromatographed by the above MPLC to furnish fractions 40–44, followed by preparative HPLC repeatedly with CH_3CN–H_2O (17:83) as the isocratic eluent system to afford compound **1** (38 mg) and compound **2** (18 mg).

3.3.1. (23S,24S)-21-hydroxymethyl-24-{[O-β-D-GLUCOPYRANOSYL-(1→4)-β-D-FUCOPYRANOSYL]OXY}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl O-(4-O-acetyl-α-L-rhamnopyranosyl)-(1→2)-O-[β-D-XYLOPYRANOSYL-(1→3)]-α-L-arabinopyranoside (1)

White amorphous solid; $[\alpha]_D^{25}$ – 40.4 (*c* = 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 254 (3.65) nm; IR (KBr) ν_{max}: 3396, 2923, 1729, 1051 cm^{-1}; HR–ESI–MS: *m/z* 1235.5327 $[M - H]^-$ (Calcd for $[C_{57}H_{87}O_{29}]^-$, 1235.5327). ^1H NMR and ^{13}C NMR spectroscopic data were in Table S1.

3.3.2. (23S,24S)-21-hydroxymethyl-24-{[O-β-D-GLUCOPYRANOSYL-(1→4)-β-D-FUCOP-YRANOSYL]OXY}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl O-(α-L-rhamnopyranosyl)-(1→2)-O-[β-D-XYLOPYRANOSYL-(1→3)-α-L-arabinopyranoside (2)

White amorphous solid; $[\alpha]_D^{25}$ –38.8 (*c* = 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 254 (3.61) nm; IR (KBr) ν_{max}: 3424, 2925, 1637, 1055 cm^{-1}; HR–ESI–MS: *m/z* 1193.5226 $[M - H]^-$ (Calcd for $[C_{55}H_{85}O_{28}]^-$, 1193.5222). ^1H NMR and ^{13}C NMR spectroscopic data were in Table S2.

3.3.3. 26-O-β-D-GLUCOPYRANOSYL-3β-hydroxy-22α-methoxyfurosta-5,25(27)-diene-1β-yl sulphate (3)

White amorphous solid; $[\alpha]_D^{25} = -31.37$ (*c* = 5.1, C_5H_5N); UV (MeOH) λ_{max} (log ε) 205 (4.33) nm; IR (KBr) ν_{max} 3433, 3342, 2949, 1242, 1067, 951, 927; HR–ESI–MS: *m/z* 701.3224 $[M]^-$ (Calcd for $[C_{34}H_{53}O_{13}S]^-$, 701.3212). ^1H NMR and ^{13}C NMR spectroscopic data were in Table S3.

3.4. Acid hydrolysis of 1–3

A solution of compound **1** (10 mg) in 1 M HCl with (dioxane–H_2O, 1:1, 6 mL) was heated at 80 °C for 2 h. After cooling, the reaction mixture was evaporated under reduced pressure to remove dioxane and diluted to 8 mL with H_2O, and then extracted with EtOAc (4 mL × 6 mL). The aqueous layer was neutralised by Ag_2CO_3 to pH 6–7 and filtered with the microporous membrane and further concentrated to an appropriate volume. Five sugars were identified as Glc, Xyl, Ara, Fuc and Rha by comparison with authentic samples (D-glucose, D-xylose, L-arabinose, D-fucose and L-rhamnose) through TLC (silica gel) detection with the solvent system EtOAc: MeOH: HAc: H_2O (20:5:3:3) and $CHCl_3$: MeOH: HAc: H_2O (16:10:3:3). In the same way, compound **2** (6 mg) was subjected to acid hydrolysis to give a sugar fraction. TLC analysis of the sugar fraction under the same condition as in the case of that of **1** showed the presence of Glc, Xyl, Ara, Fuc and Rha. Compound **3** (6 mg) was subjected to acid hydrolysis as described for **1** to give a sugar fraction. TLC analysis of the sugar fraction indicated the presence of Glc.

4. Conclusion

In summary, the present study describes the isolation and characterisation of three new steroidal saponins including two spirostanol glycosides (**1–2**) and one furostanol glycoside 1-sulphate (**3**) from *H. thibetanus*. According to previous investigations, steroidal saponins from plants are common with a double bond at C-5(6) or C-9(11), furthermore, glycosidation position is usually at 3-OH in most cases. It's worthy to note that the presence of a C-25(27) double bond and meanwhile glycosyl at 1-OH found in spirostanol glycosides **1–2** is unusual.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S27 and Tables S1–S3.

Disclosure statement

No potential conflict of interest was reported by the authors.

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