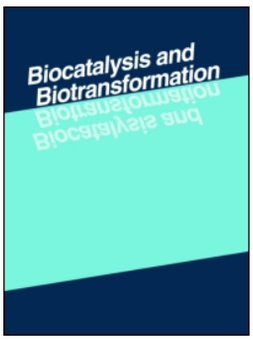

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RESEARCH ARTICLE

Biotransformation of 5-en-3β-ol steroids by *Mucor circinelloides lusitanicus*

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Abstract

In this work, we report the mode of biotransformation of 5-en-3β-ol steroids using *Mucor circinelloides lusitanicus* for the first time. Here, we selected seven 5-en-3β-ol steroids as substrates. The main characteristic of the fungus was to introduce a 7α-hydroxyl group into substrates **1**–**5**. With substrate **2**, 3β, 7α, 11α-trihydroxypregna-5-en-20-one (**2b**) was obtained as the final product in good yield (46.4%). All the metabolites were determined by infrared spectra, high-resolution mass spectrometry, proton nuclear magnetic resonance, and carbon-13 nuclear magnetic resonance.

Keywords: biotransformation, 5-en-3β-ol steroids, 7α-hydroxylation, 11α-hydroxylation, *Mucor circinelloides lusitanicus*

Introduction

The biotransformation of steroids by microorganisms has been studied extensively over recent decades (Agnieszka & Jadwiga 2007), with the most important transformations being thiose catalyzed by hydroxylases and dehydrogenases. To introduce hydroxyl groups into the steroids structure is very difficult by chemical synthesis except for the 17-position, which explains the extensive study of hydroxylation of steroids by organism. Steroid hydroxylation by microorganisms offers the possibility of obtaining both regioselective and stereoslective reactions.

It has been reported that many 7α-hydroxylated steroids show important activities such as upregulating immunity (Morfin 2002), improving memory (Yau et al. 2006), being neuronal activators (Matsunaga et al. 2004), and treating adolescent obesity (Máčová et al. 2014). Therefore, recent work points to an important role for B-ring modification, especially the 7α-hydroxylation. Although 7α-hydroxylation is the most common reaction in the biotransformation of steroids and there are a lot of reports of organisms used in the 7α-hydroxylation of steroids (Andrea et al. 2006; Schaaf & Dettner 2000; Teresa 1999), few can be readily applied on a large scale. Studies of the 7α-hydroxylation mechanism are restricted because none of the 7α-hydroxylated derivatives of 3-hydroxy-steroids is commercially available. Therefore, there is still much remaining to be learned about new biocatalytic microorganisms and increasing yield. On the other hand, 11α-hydroxylation is a commonly used reaction in the steroid industry (Petrič et al. 2010). It has been reported for several species of filamentous fungi including *Cephalosporium aphidicola* (James & Almaz 1996), *Rhizopus* spp., *Aspergillus* spp. (Petrič et al. 2010), *Curvularia* spp. (Fernandes et al. 2003), *Cunninghamella* spp. (Muhammad et al. 2005),

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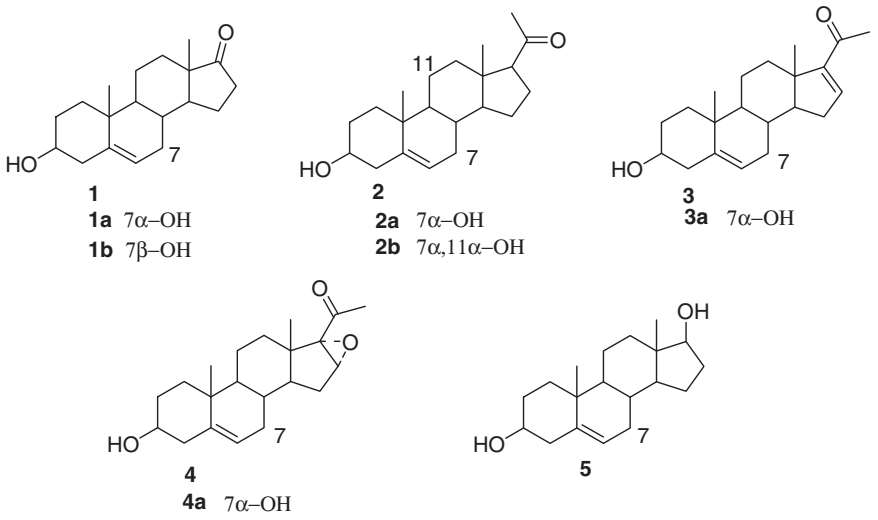

Figure 1. Structures of the substrates (**1**–**5**) and the metabolites (**1a**–**4a**).

Trichoderma hamatum (Agnieszka & Jadwiga 2007), and *Cochliobolus lunatus* (Marija et al. 1990) can introduce hydroxyl groups into the 11-position of the steroid skeleton.

Fungi are widely used in steroid biotransformations for their versatile enzymatic repertoire. Although fungi belonging to the genus *Mucor* have been applied to transform steroids (Andrew et al. 2007; Faramarzi et al. 2008; Ge et al. 2008; Wang et al. 2013), no studies have been carried out using *Mucor circinelloides lusitanicus* to transform 5-en-3β-ol steroids to date. *M. circinelloides lusitanicus* is widely distributed in nature and has considerable economic importance (María et al. 2005; Mirosława et al. 2006; Saha 2004; Tadeusz et al. 2002). We have found that the fungus *Mucor racemosus* could hydroxylate 5-en-3β-ol steroids at 7α, 7β, and 11α-positions (Ge et al. 2008), but the yield of the 11α-hydroxylated product was only 19.6%. Now we have employed *M. circinelloides lusitanicus* to transform 5-en-3β-ol steroids in order to explore the potential hydroxylation utility of this fungus.

In the present study, seven 5-en-3β-ol steroids (**1-5**, shown in Figure 1 and **6**–**7** shown in Figure 2) were selected as substrates. Substrates **1-5** can be transformed to their 7α-hydroxylated derivatives. It is interesting that the main product from **2** was 3β, 7α, 11α-trihydroxypregna-5-en-20-one (**2b**) which could be easily crystallized from the mixture with a relatively high yield (46%). This makes *M. circinelloides lusitanicus* different from its' other congeneric organism used in the transformation of 5-en-3β-ol steroids (Andrew et al. 2007; Ge et al. 2008; Madyastha & Joseph 1995, Wang et al. 2013). This allows us to prepare some new steroid compounds

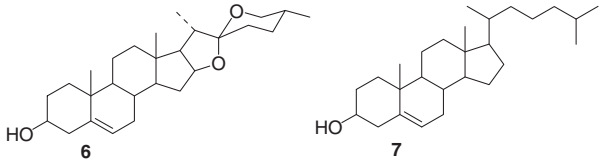

Figure 2. The structures of cholesterol (**6**) and diosgenin (**7**).

which are difficult to synthesize by chemical methods (Shan et al. 2009, 2013).

Materials and methods

Instrumental methods

Sterilization was carried out in an HVE-50 Hirayama autoclave. Aseptic operations were performed in a Class II A/B3 Biological Safety Cabinet from Forma Scientific. Incubation was carried out on a HZQ-Q orbital shaker. Transformations were monitored by thin layer chromatography (TLC) on pre-coated silica gel GF254 (0.5 mm, Qingdao Hailang, Qingdao city, China). Components were visualized by heating. Flash column chromatography (FCC) was carried out on silica gel (100∼200 mesh, Shanghai, China). Infrared (IR) spectra were recorded on a Thermo Nicolet 200 spectrometer using KBr disks in the 400∼4000 cm^{-1} region. Melting points were measured on a WC-1 melting-point apparatus and are uncorrected. proton nuclear magnetic resonance (^{1}H NMR), carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were obtained on a Bruker DPX400 instrument with Me_4Si as internal standard, and the chemical shifts are given in δ values. High resolution–electron spray

mass spectra were recorded on a Waters Q-Tof MS System in positive ion mode. Starting materials were obtained commercially (Hanzhong Hanjiang-Zhenhua Bio-Tec. LTD., Hanzhong, China). Optical rotations were determined on a Perkin Elmer 341 polarimeter at 20°C in MeOH. Single crystal structure was carried out on a Rigaku R-AXIS-IV area detector.

Microorganism

The organism used in the present study was isolated from soil of the north suburb of Zhengzhou, People's Republic of China, and identified as *M. circinelloides lusitanicus* by the company of Microbial ID (Newark, NJ). The fungus was maintained on potato dextrose agar (PDA) slants at 4°C.

Conditions and transformation

The optimized liquid medium consisted of maltose 4.3%, peptone 2.3%, yeast extract 0.1%, K_2HPO_4 0.9‰, $MgSO_4 \bullet 7H_2O$ 0.9‰ and pH 6.0. Flasks (500 ml) containing 100 ml medium were inoculated with spore suspension (1.0 ml, 2.2×10^8 counts) from a 2-day-old culture grown on PDA slants and incubated at 28°C on a rotary shaker (210 rpm) for 48 h. Then, 100 mg of a 5-en-3β-ol steroid substrate dissolved in 1 ml acetone or dimethyl sulfoxide was added to the flask. Incubation was continued for 4 days.

Isolation of transformation products

After incubation, the fermentation media and the mycelium were extracted separately with ethyl acetate. Since the mycelial extract contained only the added substrate, it was not processed further. The media extracts were washed three times each by saturated $NaHCO_3$, brine, and distilled water, respectively and dried with anhydrous sodium sulfate. After evaporation of the solvent, the mixed products were analyzed by TLC in ethyl acetate/chloroform (1:1) and separated by silica gel chromatography using the same solvents as eluant. Product **2b** could be obtained by recrystallizing the extract mixture with methanol and chloroform (1:3).

Time course experiment

The time course of the experiment was conducted as a guide to the sequence of reactions. Freshly obtained spores from peptone dextrose agar slopes were transferred aseptically into three Erlenmeyer flasks (250 ml) containing 50 ml liquid sterile fermentation medium and incubated on a rotary shaker at 210 rpm and 30 °C for 2 days. Then, 150 mg of substrate **2** was dissolved in DMSO and added to each fermentation flask. The fermentations were continued and 3 ml broth of every flask harvested every 24 h over 7 days and extracted with the same volume of ethyl acetate. Then, the solvent was evaporated under vacuum to give the reaction mixtures.

Extracted reaction mixtures were dissolved in CH_3OH (3 ml) and then analyzed by high-performance liquid chromatography (20 μl). Each analytical determination was performed in triplicate. The mobile phase was CH_3OH/water (70:30, v/v) at a flow rate of 1 ml/min, run over 15 min. UV detection was determined at 205.0 nm.

Results

Identification of metabolites of 1

*3β, 7α-Dihydroxyandrost-5-en-17-one (**1a**)*
Colorless crystals (crystallized from methanol); Yield: 43.6%; mp: 179.3∼181.2°C (literature 181.5∼183.5°C) (Crabb et al. 1980); $[\alpha]_D^{20}$-71° (c.0.21, MeOH); IR (KBr)ν_{max}: 3431, 2933, 1730, 1657, 1621, 1375, 1057 cm^{-1}; ^1H NMR (MeOD): 0.89 (3H, s, H-18), 1.02 (3H, s, H-19), 3.46 (1H, m, H-3), 3.89 (1H, t, H-7, $^2J = 7.2$ Hz), 5.57 (1H, dd, H-6, $J = 1.4, 5.2$ Hz); ^{13}C NMR data (shown in Table 1); MS: m/z: 305.2112 $[M+H]^+$ (calcd. 305.2117) (Supplement Figures HNMR-1a-1 and HNMR-1a).

*3β, 7β-Dihydroxyandrost-5-en-17-one (**1b**)*
Colorless crystals (crystallized from methanol); Yield: 12.6%; mp: 208.6∼209.8°C (literature 215∼216°C) (Crabb et al. 1980); $[\alpha]_D^{20}$-93° (c.0.29, MeOH); IR (KBr)ν_{max}: 3364, 2930, 1730, 1660, 1629, 1380, 1056 cm^{-1}; ^1H NMR (DMSO-d_6): 0.78 (3H, s, H-18), 0.97 (3H, s, H-19), 3.26 (1H, m, H-3), 3.70 (1H, d, H-7, $J = 8.0$ Hz), 5.18 (1H, s, H-6); ^{13}C NMR data (shown in Table 1); MS: m/z: 305.2115 $[M+H]^+$ (calcd. 305.2117) (Supplement Figures HNMR-1b-1 and HNMR-1b).

Identification of metabolites of 2

*3β, 7α-Dihydroxypregna-5-en-20-one (**2a**)*
Colorless crystals (crystallized from methanol); Yield: 24.4%; mp: 178.2∼180.7 °C (literature 180∼182°C) (Baulieu et al. 2004); $[\alpha]_D^{20}$-49° (c.0.20, MeOH); IR (KBr)ν_{max}: 3420, 2935, 1698, 1661, 1358, 1054 cm^{-1}; ^1H NMR ($CDCl_3$): 0.64 (3H, s, H-18), 1.00 (3H, s, H-19), 2.14 (3H, s,

Table I. ^{13}C NMR spectral data for **1a**∼**4a** (100.6 M Hz, 25°C, TMS).

Carbon	**1a**[a]	**1b**[c]	**2a**[b]	**2b**[c]	**3a**[c]	**4a**[c]
1	38.6	36.8	37.0	38.4	36.3	36.9
2	32.1	31.3	31.6	31.6	31.2	31.5
3	71.9	71.3	71.3	70.2	69.6	70.2
4	42.9	41.9	42.2	42.8	42.0	42.5
5	146.8	141.6	146.3	144.6	143.9	144.4
6	124.6	127.1	123.7	124.3	124.2	124.7
7	64.9	70.0	65.2	63.5	63.4	63.6
8	38.6	39.3	37.5	37.1	35.4	35.8
9	46.4	48.2	41.9	47.8	41.5	39.4
10	38.0	36.3	37.4	38.5	36.8	37.4
11	21.2	20.2	20.7	67.2	19.8	20.2
12	32.4	31.7	38.2	49.1	34.1	31.7
13	43.8	47.3	43.8	43.6	45.2	41.2
14	49.6	50.9	49.7	49.5	49.5	42.1
15	22.7	24.2	24.4	23.7	31.7	27.2
16	36.6	35.7	22.9	22.6	145.7	60.8
17	223.9	220.5	63.5	62.8	154.2	70.8
18	13.7	13.4	13.0	14.1	15.4	15.2
19	18.6	19.0	18.2	17.7	17.7	18.2
20			209.6	208.7	196.1	205.3
21			31.3	31.3	31.3	26.2

[a]MeOD.
[b]$CDCl_3$.
[c]DMSO-d_6.

H-21), 2.59 (1H, t, H-17, $^2\mathcal{J}=9.2$ Hz), 3.59 (1H, m, H-3), 3.87 (1H, bs, H-7), 5.62 (1H, dd, H-6, $\mathcal{J}=1.2$ 5.2 Hz); ^{13}C NMR data (shown in Table I); MS: m/z: 333.2424 $[M+H]^+$ (calcd. 333.2430) (Supplement Figures HNMR-2a).

*3β, 7α, 11α-Trihydroxypregna-5-en-20-one (**2b**)*
Colorless needle crystals (crystallized from methanol); Yield: 46.4%; mp: 242.8∼243.8°C (literature 255∼257°C) (Baulieu et al. 2004); $[\alpha]_D^{20}$-40.2° (c.0.99, MeOH); IR (KBr)ν_{max}: 3328, 2969, 2936, 1700, 1357, 1046 cm^{-1}; ^{1}H NMR (DMSO-d_6): 0.50 (3H, s, H-18), 1.00 (3H, s, H-19), 2.07 (3H, s, H-21), 2.59 (1H, t, H-17, $^2\mathcal{J}=9.1$ Hz), 3.34 (1H, m, H-3), 3.57 (1H, d, H-7, $\mathcal{J}=8.5$ Hz), 3.80 (1H, m, H-11), 5.44 (1H, d, H-6, $\mathcal{J}=5.3$ Hz); ^{13}C NMR data (shown in Table I); MS: m/z: 371.2208 $[M+Na]^+$ (calcd. 371.2198). For X-ray diffraction analysis is shown in Figure 3(Supplement Figure HNMR-2b).

Identification of metabolites of 3

*3β, 7α-Dihydroxypregna-5, 16-diene-20-one (**3a**)*
White powder; Yield: 10.6%; mp: 189∼191°C; $[\alpha]_D^{20}$-74.1° (c.0.50, MeOH) (Gao et al. 2011); IR (KBr)ν_{max}: 3480, 2934, 1660, 1588, 1372, 1055 cm^{-1}; ^{1}H NMR (DMSO-d_6): 0.83 (3H, s, H-18), 0.93 (3H, s, H-19), 2.21 (3H, s, H-21), 3.33 (1H, m, 3-H), 3.69 (1H, bs, 7-H), 5.43 (1H, d, H-6, $\mathcal{J}=4.9$ Hz), 6.91 (1H, s, 16-H); ^{13}C NMR data (shown in Table I); MS: m/z: 353.2087 $[M+Na]^+$ (calcd. 353.2093) (Supplement Figure HNMR-3a).

Identification of metabolites of 4

*3β, 7α-Dihydroxypregna-16α, 17α-epoxy -5-en -20-one (**4a**)*
Colorless crystals (crystallized from methanol); Yield: 14.7%; mp: 185∼186°C (literature 185∼186°C) (Ge et al. 2008); $[\alpha]_D^{20}$-64° (c.0.20, MeOH); IR (KBr)ν_{max}: 3430, 2955, 1720, 1644, 1376 cm^{-1}; ^{1}H NMR (DMSO-d_6): 1.01 (3H, s, H-18), 1.06 (3H, s, H-19), 2.08 (3H, s, H-21), 3.18 (1H, m, 3-H), 3.68 (1H, s, 7-H), 4.02 (1H, s, 16-H), 5.51 (1H, d, H-6, $\mathcal{J}=4.8$ Hz); ^{13}C NMR data (shown in Table I); MS: m/z: 369.2036 $[M+Na]^+$ (calcd. 369.2042) (Supplement Figure HNMR-4a).

Discussion

All products of the transformations were identified through IR, ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry analysis. The positions and configurations of the hydroxyl group introduced were confirmed mainly from the δ changes in NMR spectra relative to the starting material and data reported in the literature. X-Ray diffraction analysis was carried out to establish the axial (α) orientation of C7 and C11 hydroxyl group in compound **2b** shown in Figure 3.

To clarify whether the microorganism had the ability to hydroxylate 5-en-3β-ol steroids, two representative compounds, i.e. dehydroepiandrosterone (**1**) and pregnenolone (**2**) were used as substrates. By biotransformation, 7α, 7β, or 11α monohydroxy or dihydroxy products were obtained. 3β, 7α-Dihydroxyandrost-5-en-17-one (**1a**) and 3β, 7α, 11α-trihydroxypregna-5-en-20-one (**2b**) were the main products of substrates **1** and **2**, respectively. These results indicated that 7α-hydroxylation was a characteristic feature of *M. circinelloides lusitanicus* in transforming 5-en-3β-ol steroids. We utilized the microorganism to transform 3β-Hydroxypregna-5, 16-diene-20-one (**3**) and 3β-Hydroxypregna-16α, 17α-epoxy-5-en-20-one (**4**), and found that they were also hydroxylated at the 7α-position. However, the yields of **3a** and **4a** were much lower than that of **1a**. The double bond and the epoxy group seemed to hinder the activity of the hydroxylase. If androstenediol (**5**) was added to the medium of the matured fungi, the products **1a** and **1b** could be obtained with yields of 8.9% and 11.1% respectively, which were much lower than that of **1**. This revealed that *M. circinelloides lusitanicus* could be induced to produce a 17-oxidase, but the complex transformation meant the yield of **1a** and **1b** was very low. When cholesterol (**6**) and diosgenin (**7**) (Figure 2) were fed to the broth of the mature organism separately, the original material was unchanged. We speculate that the large

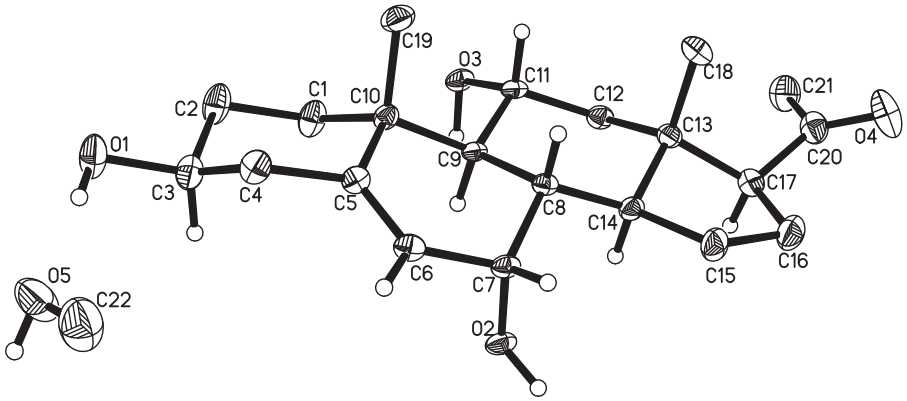

Figure 3. The X-ray diffraction analysis diagram of **2b**.

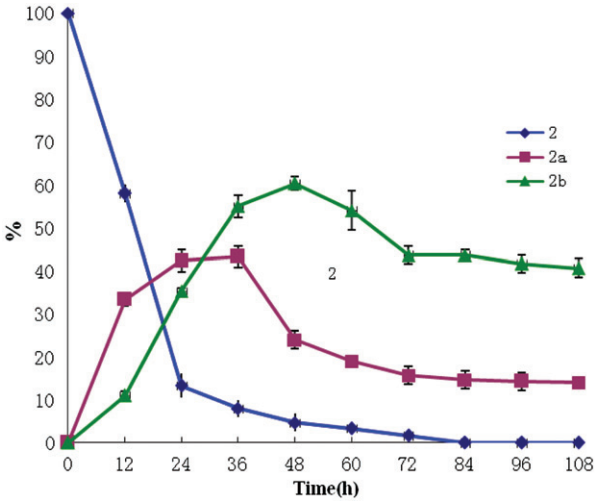

Figure 4. Time course for the biotransformation of pregnenolone (2) by *Mucor circinelloides lusitanicus*.

group at C17 in the structures of **6** and **7** made them unsuitable for the active site of the hydroxylase (Peart et al. 2011). Some investigations have reported that some *Mucor* sp. can isomerize 5-en-3-ol steroids to 4-en-3-one structures or cleave the C17 side-chain. *M. circinelloides lusitanicus* appears to lack the isomerase and could not cleave the side-chain of the selected substrates **2**–**4** and **6**-**7** (Hunter et al. 2009).

The ability of *M. circinelloides lusitanicus* to transform 5-en-3β-ol steroids is similar to *M. racemosus* and *Mucor piriformis*, but *M. circinelloides lusitanicus* is more useful in transforming **2**. Substrates **1** and **3**–**5** could not be dihydroxylated over the same transforming period, which revealed that the specific structure of **2** was more suitable for the hydroxylase of *M. circinelloides lusitanicus*. The product **2b** is not commercially available, hindering further studies on structural modification and evaluation of activity. So we optimized the original transformation conditions and the medium components to improve the yield of **2b**. With the optimized conditions, **2b** could be obtained in much higher yield (46.4%) than reported for *M. racemosus* (19.6%) (Ge et al. 2008) and *M. piriformis* (35.0%) (Madyastha & Joseph 1995).

The time course of the biotransformation of pregnenolone (**2**) was also studied (shown in Figure 4). From the time course, we concluded that the 7α-hydroxylation took place rapidly within 24 h, then the 11α-hydroxyl group was introduced subsequently. After 36 h, the concentration of monohydroxylated metabolite had declined and the dihydroxylated metabolite accumulated to a maximum at 48 h then slightly declined to around 46% at 96∼108 h. Feeding **2a** to mature fungi, produced **2b** in the fermentation broth after 12h, further confirming that **2a** is an intermediate and **2b** is the finally product.

It is known that compound **1** is a major hormone in human nerve tissues, and its' therapeutic role in repairing neurons is well documented. Recent studies indicated that hydroxylated metabolites of **1** have many profound activities (Matsunaga et al. 2004; Yau et al. 2006). So, using *M. circinelloides lusitanicus*, we have generated some structural modification and evaluated bioactivity, which showed that some of the benzylidene derivatives of **1a** and **2b** have remarkable activity against EC109 cells (Shan et al. 2009, 2013). This needs to be further studied.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online