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Identification and Synthesis of Impurities in Pinocembrin—A New Drug for the Treatment of Ischemic Stroke†

Yang, Qingyun(杨庆云) Tong, Yuanfeng(童元峰) Chen, Feng(陈锋) Qi, Yan(戚燕) Li, Wei(李薇) Wu, Song*(吴松)

State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

Four minor impurities in pinocembrin (**1**)—a new drug to treat ischemic stroke, were analysed and identified by means of HPLC-UV-MS analysis, spectroscopic evidences and chemical synthetic methods. Their chemical structures were identified as 5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (**2**), 3-phenyl-1-(2,4,6-trihydroxyphenyl)-1-propanone (**3**), 5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one (**4**), and 2,3-dihydro-5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one (**5**), respectively. All of the impurities were side products of excessive hydrogenation of the target product **1** or the starting material **2** in the course of synthesis, and **5** was a new compound.

Keywords pinocembrin, impurity, ischemic stroke, side product, synthesis

Introduction

Ischemic stroke has become an increasingly severe medical and social problem with high attach and fatality rate, due to rapid growth of aging people populations.[1] It has been estimated that about six million people have died from stroke in 2005 and that more than 90 percent of these decedents will have occurred in less affluent countries.[2] Stroke patients must not only survive the acute stages of infarction, but must cope with significant mental, physical and economic stresses associated with neurological impairment. Considering the cost in loss of life, physical and mental disability, the need for effective therapeutic interventions is obvious.[3]

2,3-Dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (**1**), well known under the generic name pinocembrin, was isolated from *propolis* for the first time. Previous investigations have demonstrated that pinocembrin exhibited antibiotic,[4] antioxidant and cytotoxic activities,[5-7] whereas our study found this compound showed potent protective effect on neurovascular unit. Pinocembrin induced relaxation of rat aortic rings through an endothelium-dependent and independent pathway, this compound also could improve rat cognitive impairments induced by chronic cerebral hypoperfusion.[8] It is promising that pinocembrin could be developed as a new drug to treat ischemic stroke, and it is now in phase I clinical trial. Nowadays impurities are sure to affect the drug qualities seriously, which will eventually reflect on people's health. Therefore, investigation on the impurities in drug is an important and meaningful research field.

Pinocembrin **1** was prepared generally from 5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (**2**) upon treatment with hydrogen and palladium in ethanol (Scheme 1).[9] As a part of our research on the drug impurities, we analyzed the pinocembrin sample on HPLC, and four unconspicuous impurity peaks were found. On the basis of HPLC-UV-MS analysis, we further analyzed their contents and possible structures. Their structures were eventually identified by the method of isolation and synthesis. All of the impurities were determined as byproducts of excessive hydrogenation of pinocembrin or the starting material in the course of the synthesis, and impurity **5** was identified as a new chromone derivative. Here we wish to report the analysis, isolation, synthesis and structural elucidation of these impurities in pinocembrin substances.

Experimental

Chemicals, reagents and apparatus

Solvents and reagents (K_2CO_3, KOH) for reactions and column chromatography (CC) were purchased from Beijing Beihua Fine Chemicals Co., Ltd. Silica gel (GF_{254} 100—200 meshes) for CC were obtained from Qingdao Marine Chemical Factory, Qingdao, Shandong Province, China. 10% Pd/C, cyclohexanecarboxylic acid chloride and 2',4',6'-trihydroxyacetophenone were purchased from Acros Organics (New Jersey, USA). Pino-

* E-mail: ws@imm.ac.cn; Tel.: 0086-010-83163542; Fax: 0086-010-63017757
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cembrin substances (20060901) were provided by Beijing Collab Pharma Co. Ltd. IR spectra were recorded on a IR-47 spectrometer. Melting points were determined with a YRT-3 apparatus (Tianjin University Precision Apparatus Factory, Tianjin, China). NMR spectra were recorded on a Varian MERCURY-400 spectrometer using tetramethylsilane (TMS) as internal standard and deuterated dimethylsulfoxide (DMSO-d_6) as solvent. Chemical shifts were given on the δ-scale.

High performance liquid chromatography

HPLC analysis was performed on a Shimadzu liquid chromatography LC-10ATvp equipped with SPD-M10AT VP UV-Vis spectrophotometric detector (Shimadzu Co., Japan). The analysis was carried out on an ODS column (Apollo C18, 150×4.6 mm I.D., 5 μm, Grace) maintained at temperature 25 ℃. Mobile phase A was a phosphate buffer, adjusted to pH 3.0 and methanol used as mobile phase B. Mobile phase was A and B mixed in the ratio of 64∶36 (*V*/*V*). Flow rate was kept as 1.0 mL/min, injection volume was 20 μL, chromatographic data acquisition time for 45 min and UV detection was carried out at 290 nm.

HPLC-ESI-MS and HPLC-UV

HPLC-UV and HPLC-ESI-MS analysis were carried out on an Agilent 1100 series LC/MSD Trap equipped with UV-VIS spectrophotometric detector (Agilent Co., Santaclara, CA, USA). Analyst software was used for data acquisition and data processing. The turbo ion spray voltage was maintained at 5.5 kV and temperature was set at 350 ℃. The auxiliary gas and curtain gas was high purity nitrogen. Zero air was used as the nebulizer gas. LC-MS spectra were acquired from *m*/*z* 100—600 in 0.1 amu steps with 2.0 s dwell time. The analysis was carried out using a column (Apollo C18, 150 mm×4.6 mm I.D., 5 μm, Grace) and the same analytical method mentioned above.

Synthesis of 3-phenyl-1-(2,4,6-trihydroxyphenyl)-1-propanone (3)

Compound **2** (15.0 g, 0.059 mol) in ethyl acetate (1400 mL) was hydrogenated with 10% Pd-C (2.5 g) under H_2 at 60 ℃ for 12 h. After cooling, the reation mixture was filtered and concentrated *in vacuo* to give a residue, which was recrystallized in hexane/ethyl acetate (3∶1, *V*/*V*) to yield compound **3** (12.2 g) as a light-yellow solid in 80% yield, m.p. 160 ℃. Its ^{1}H NMR data (DMSO-d_6, 400 MHz) see Table 1. ESI-MS *m*/*z*: 259.2 $[M+H]^+$. These data were in agreement with those reported in the literature.[10]

Isolation and synthesis of 5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one (4)

Isolation of compound 4 Compound **2** (100.0 g, 0.39 mol) was hydrogenated for 5 h using the same method to synthesize crude product **1** (100.0 g), which was recrystallized in ethyl acetate for five times. The mother liquid was concentrated *in vacuo* to yield a solid, which was analysed by HPLC and impurity **4** was observed around 30%. Then, the solid was purified on silica gel column with a mixture of CH_2Cl_2/CH_3OH (200∶1, *V*/*V*) as eluent to obtain **4** (0.5 g) as a white amorphous powder in 0.5% yield, m.p. 214 ℃. Its ^{1}H NMR data (DMSO-d_6, 400 MHz) see Table 1. ESI-MS *m*/*z*: 261.2 $[M+H]^+$. These data were in agreement with those reported in the literature.[11]

Synthesis of compound 4 To a solution of 2',4',6'-trihydroxyacetophenone (**6**, 2.3 g, 0.013 mol) in acetone (150 mL), anhydrous K_2CO_3 (20 g, 0.188 mol) was added, and the solution was stirred for 1 h at room temperature. Then cyclohexanecarboxylic acid chloride (10 mL, 0.039 mol) was added dropwise, and the mixture was refluxed for 10 h. The reaction mixture was

Table 1 Comparative ^{1}H assignments for compounds **1**, **2**, **3**, **4**, and **5**

No.	**1**	**2**	**3**	**4**	**5**
2	5.58 (dd, 1H, *J*=2.8, 12.0)	—	—	—	4.18—4.23 (m, 1H)
3	2.78 (dd, 1H, *J*=2.8, 16.0) 3.37 (dd, 1H, *J*=4.0, 12.0)	6.97 (s, 1H)	5.80 (brs, 2H)	6.08 (s, 1H)	2.53—2.80 (m, 2H)
5	—	—		—	—
6	5.89 (d, 1H, *J*=2.0)	6.22 (brs, 1H)	—	6.15 (d, 1H, *J*=2.0)	5.82 (brs, 2H)
8	5.92 (d, 1H, *J*=2.0)	6.52 (brs, 1H,)	3.28 (t, 2H, *J*=7.2)	6.32 (d, 1H, *J*=2.0)	
9	—	—	2.87 (t, 2H, *J*=7.2)	—	—
1'	—	—	—	2.51—2.57 (m, 1H)	1.87—1.90 (m, 1H)
2', 6'	7.51 (d, 2H, *J*=7.2)	8.05 (d, 2H, *J*=8.0)	7.16—7.26 (m, 5H)	1.18—1.92 (m, 10H)	1.02—1.74 (m, 10H)
3', 4', 5'	7.37—7.44 (m, 3H)	7.57—7.59 (m, 3H)			
4-OH	—	—	10.34 (s, 1H)	—	—
2,6-OH	—	—	10.22 (s, 2H)	—	—
5-OH	12.12 (s, 1H)	12.84 (s, 1H)	—	12.80 (s, 1H)	12.10 (s, 1H)
7-OH	10.81 (s, 1H)	10.90 (s, 1H)	—	10.79 (s, 1H)	10.71 (s, 1H)

[a] Data were measured in DMSO-d_6 at 400 MHz.

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cooled to room temprature, filtered and concentrated *in vacuo* to furnish a solid, which was dissolved in EtOAc (100 mL) and washed with water (50 mL×3). The combined organic layer was evaporated under vacuo to obtain an orange solid. The solid was dissolved in 5% KOH/EtOH (250 mL) and refluxed for 15 h. The reaction mixture was cooled, evaporated and water (50 mL) was added. The mixture was extracted with EtOAc (50 mL×3). The combined organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuo to get a residue, which was purified by chromatography on silica gel using a mixture of CH_2Cl_2/CH_3OH (200∶1, *V*/*V*) as eluent to give product **4** (2.3 g) as a white solid in 67% yield, m.p. 212 ℃. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.22—1.93 (m, 10H, cyclohexyl-CH_2), 2.53—2.58 (m, 1H, H-1'), 6.08 (s, 1H, H-3), 6.16 (s, J=2.0 Hz, H-6), 6.32 (d, 1H, J=2.0 Hz, H-8), 10.79 (s, 1H, OH-7), 12.80 (s, 1H, OH-5). ESI-MS *m*/*z*: 261.1 $[M+H]^+$. HR-ESI-MS calcd for $C_{15}H_{16}O_4$ 261.2924, found 261.2936 $[M+H]^+$. These data were in agreement with those isolated from crude product.

Synthesis of 2,3-dihydro-5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one (5)

The solution of **4** (15.0 g, 0.057 mol) in anhydrous ethanol (1400 mL) was stirred with 10% Pd-C (2.5 g) under H_2 (4 atm) at room temperature for 24 h. The reaction mixture was filtered and concentrated *in vacuo*. The obtained residues were subjected to dry flash column (petroleum ether/ethyl acetate=25∶1, *V*/*V*) to get compound **5** (12.2 g) as a white solid in 81% yield, m.p. 169.6—169.9 ℃. UV-vis (MeOH) λ_{max}: 213, 226 (sh), 289, 322 (sh) nm. ^{1}H NMR (DMSO-d_6, 400 MHz) see Table 1. ^{13}C NMR (DMSO-d_6, 400 MHz) δ: 196.7 (C-4), 166.4 (C-7), 163.3 (C-5), 163.0 (C-1a), 101.7 (C-4a), 95.5 (C-6), 94.7 (C-8), 80.9 (C-2), 40.9 (C-1'), 38.3 (C-3), 27.5 (C-2', C-6'), 25.8 (C-3'), 25.4 (C-5'), 25.3 (C-4'). IR (KBr) *v*: 2926, 1629, 1584, 1488, 1302, 1146, 1089, 835, 708, 553 cm^{-1}; ESI-MS *m*/*z*: 263.2 $[M+H]^+$. HR-ESI-MS calcd for $C_{15}H_{19}O_4$ 263.1283, found 263.1284.

Results and Discussion

Detection and identification of impurities

In the course of HPLC analysis on pinocembrin substances using analytical conditions, four inconspicuous peaks due to impurities except for pinocembrin **1** (t_R= 9.3 min) were observed at the relative retention times of 10.4 min (**3**), 12.5 min (**2**), 21.7 min (**4**) and 28.1 min (**5**). Their contents were analysed as 0.078% (**3**), 0.013% (**2**), 0.347% (**4**) and 0.288% (**5**) (HPLC-purity), respectively (Figure 1). In order to clarify these impurities, we further researched the substances. Comparison of retention time (t_R) values on HPLC of these four impurities with that of the reactant in the preparation of pinocembrin revealed that impurity **2** (t_R=12.5 min) was the starting material. But compounds **3**, **4**, and **5** were unknown impurities, which did not exist in the starting material.

In order to determine the structures of these compounds, the same samples were subjected to HPLC-ESI-MS analysis using HPLC-ESI-MS conditions to identify the mass of the impurities. In positive ion model of HPLC-ESI-MS, compounds **1**, **4**, and **5** showed their ion peaks at *m*/*z* 257.1 $[M+H]^+$, *m*/*z* 261.2 $[M+H+4H]^+$, and *m*/*z* 263.2 $[M+H+6H]^+$ respectively (Figures 2, 3), which suggested that these impurities could be side products of excessive hydrogenation of pinocembrin or its synthetic material in the course of the synthesis of pinocembrin.

Isolation and synthesis of impurities

In order to confirm the above assumption, it is necessary to obtain enough impurities. Therefore, we extended the catalytic hydrogenation time from 5 h to 12 h in the course of the pinocembrin synthesis (Scheme 1). HPLC analysis of the reaction products showed the content of **3** was above 95%, while **4** and **5** were not

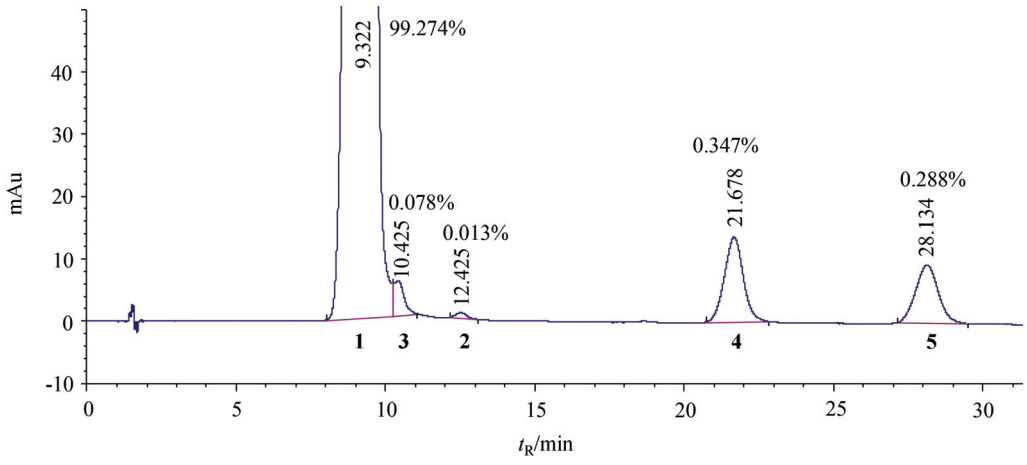


Figure 1 HPLC chromatogram of pinocembrin (**1**) (t_R=9.3 min), impurities **2** (t_R=12.5 min), **3** (t_R=10.4 min), **4** (t_R=21.7 min) and **5** (t_R=28.1 min). Mobile phase: methanol∶phosphate buffer, 64∶36 (*V*/*V*), pH 3.0; column temperature, 25 ℃.

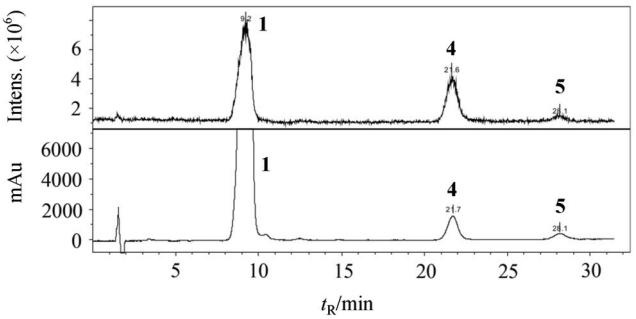


Figure 2 HPLC chromatogram and ionic current of pinocembrin (**1**) and impurities (**4**, **5**)

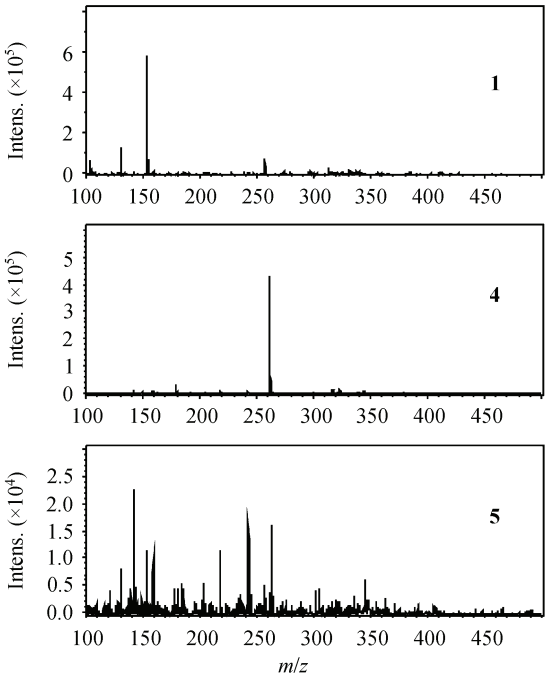
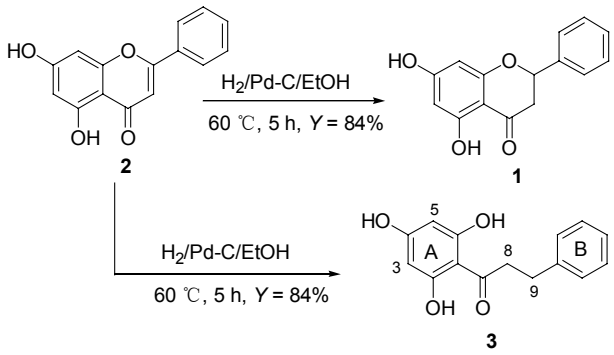


Figure 3 HPLC-ESI-MS of pinocembrin (**1**) (*m*/*z* 257.1 $[M+H]^+$), impurities **4** (*m*/*z* 261.2 $[M+H]^+$) and **5** (*m*/*z* 263.2 $[M+H]^+$).

detected. The reaction mixture was recrystallized in hexane/ethyl acetate to obtain **3**, which was identified as 3-phenyl-1-(2,4,6-trihydroxyphenyl)-1-propanone on the basis of NMR spectral analysis. Thus, impurity **3** was determined as a side product of excessive hydrogenation of pinocembrin.

Subsequently, the crude products reacted for 5 h were recrystallized in ethyl acetate, the mother liquid was observed to contain 32% impurity **4** in HPLC analysis. The solution was concentrated and further purified by silica gel column chromatography to give compound **4**. Its HR-ESI-MS showed the ion peak at *m*/*z* 261.2936 $[M+H]^+$, indicating the molecular formula of $C_{15}H_{16}O_4$ (calcd for $C_{15}H_{16}O_4$, 260.2924). In combination with ^{1}H NMR spectral data, the structure of **4** was elucidated as 5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one. It could be a side product of excessive hydrogenation of the starting material **2**. The results were in agreement with the deduce from HPLC-ESI-MS.

Scheme 1



Unfortunately, neither excessive hydrogenation nor recrystallization was helpful to get enough amount of compound **5**. So HPLC-MS and HPLC-UV analysis were used to elucidate the structure. Its HPLC-ESI-MS (Figure 3) in positive ionization mode showed the ion peak at *m*/*z* 263.2 $[M+H]^+$, suggesting the molecular formula of $C_{15}H_{18}O_4$. The UV spectrum (λ_{max} 289 and 322 nm, sh) of **5** (Figure 4) in HPLC-UV was similar as those of **1**, suggesting the presence of a dihydrochromone skeleton in **5**.[7] Combined with the difference of the HPLC-ESI-MS data between **4** (*m*/*z* 261.2 $[M+H]^+$) and **5** (*m*/*z* 263.2 $[M+H]^+$), the structure of **5** was determined as 2,3-dihydro-5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one, which was identified as a new compound.

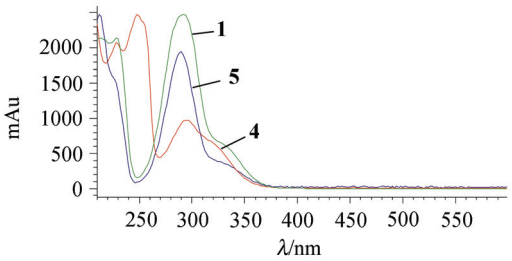


Figure 4 UV apex spectrum peaks of compounds **1**, **4**, and **5**.

In order to further confirm the structures, we employed Baker-Venkataraman transformation to synthesize **4** and **5** from the starting material 2',4',6'-trihydroxyacetophenone (**6**, Scheme 2).[13] **6** was treated with cyclohexanecarboxylic acid chloride in acetone through Baker-Venkataraman transformation to afford flavone derivative **7**, which was hydrolyzed in alkaline condition to furnish **4** in 67% yield. Eventually, **4** was further hydrogenation in the presence of 10% pd/C to get compound **5** in 81% yield successfully. Moreover, NMR experiments showed that the ^{1}H NMR data of synthetic compounds **3**, **4**, and **5** were in agreement with the isolated ones. Co-injection of the synthetic compounds **3**, **4**, and **5** into HPLC with the pinocembrin sample further confirmed the above deduce. Accordingly, impurities **3**—**5** were identified as side product of excessive hydrogenation during the synthesis of pinocembrin.

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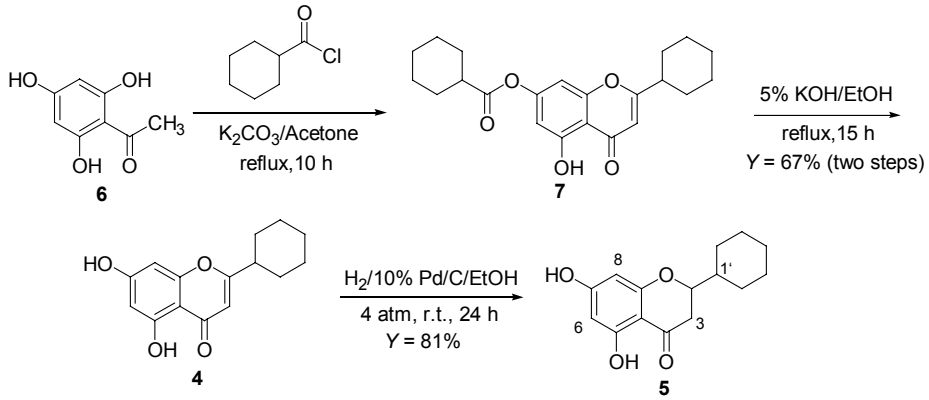
Scheme 2



Structural elucidation of impurities

ESI mass spectrum of impurity **3** in positive ion mode displayed a quasi molecular ion peak at *m*/*z* 259.2 $[M+H]^+$, which was 2 amu more than that of **1** protonated molecular ion. Compared with **1**, the ^1H NMR spectrum of **3** is two protons more than that of **1** (Table 1), indicating that **3** should be a ring cleavage product of **1** through hydrogenation in the course of synthesis. Thus, **3** was identified as 3-phenyl-1-(2,4,6-trihydroxyphenyl)-1-propanone.

HR-ESI-MS of impurity **4** gave the quasi molecular ion peak at *m*/*z* 261.2936 $[M+H]^+$, indicating the molecular formula of $C_{15}H_{16}O_4$ (calcd for $C_{15}H_{16}O_4$ 260.2924), which was 6 amu more than that of the starting material **2** ($C_{15}H_{10}O_4$, 254.2). Comparing the ^1H NMR spectrum with **2** (Table 1), 5 aromatic proton signals (δ_H 7.58—8.05) in **2** were replaced by 11 aliphatic hydrogens (δ_H 1.18—2.57) in **4**, which suggested that **4** should be a product of full hydrogenation of an aromatic ring in **2**. Accordingly, the structure of **4** was deduced as 5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one.

HR-ESI-MS of impurity **5** showed the quasi ion peak at *m*/*z* 263.1284, corresponding to the molecular formula of $C_{15}H_{19}O_4$, indicating that the structure of **5** was 2 protons more than that of **4**. In ^1H NMR spectra of **4** and **5** (Table 1), an olefinic hydrogen in **4** (δ_H 6.08) was substituted by 3 aliphatic hydrogens in **5** (δ_H 2.53—4.20), indicating that **5** should be a product of hydrogenation of double bond in **4**. The ^{13}C NMR, IR and UV spectra of **5** further confirmed the above hypothesis. Therefore, impurity **5** was determined as 2,3-dihydro-5,7-dihydroxy-2-cyclohexyl-4*H*-1-benzopyran-4-one, which was a new compound.

The structures of impurities **3**, **4**, and **5** were further confirmed by chemical synthetic methods, and all of them were determined as side products of excessive hydrogenation of the target product **1** or the starting material **2** in the course of synthesis.

Conclusions

In summary, applying HPLC-UV-MS technique, together with spectral analysis and classical synthetic methods, we analyzed and identified the structures of four impurities in a new drug—pinocembrin. All of them were determined as side products of excessive hydrogenation during the synthesis of pinocembrin. These information will direct us to prepare enough amount of impurities for pharmacological and toxicological test subsequently, and will lay a foundation for the new drug development. This investigation also demonstrated that HPLC-MS and HPLC-UV are convenient methods for providing information of unknown structures without isolation.

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(Pan, B. Lu, Z.)

