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Lignans from *Torreya jackii* identified by stopped-flow high-performance liquid chromatography–nuclear magnetic resonance spectroscopy

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Abstract

Coupled reversed-phase HPLC–NMR spectroscopy has been applied to the rapid detection and identification of plant metabolites of *Torreya jackii*, a species of Taxaceae. Analysis consisted of gradient HPLC elution and directly coupled ^{1}H NMR (500 MHz) spectroscopic detection in a stopped-flow mode. Seven lignans were detected and their structures were elucidated, based on their HPLC–^{1}H NMR spectra and MS data. The structures were confirmed by isolation of the single components followed by conventional NMR measurements.

Keywords: *Torreya jackii*; Nuclear magnetic resonance spectroscopy; Lignans

1. Introduction

Unambiguous identification of plant metabolites can usually only be accomplished by spectroscopic characterization, especially by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Traditionally this has necessitated the time-consuming isolation and purification of the metabolites from chemically most heterogenous crude extracts followed by conventional spectroscopic characterization (off-line measurement). However, methodologies for rapid identification of natural products have been more developed in recent years. Recent advances in the combination of high-performance liquid chromatography (HPLC) with MS demonstrated the usefulness of recording mass spectra without isolation of individual compounds [1]. Although to some extent HPLC–MS has simplified the steps of metabolite identification, on most occasions MS data alone are insufficient for structure elucidation of plant secondary products. Further data, especially NMR spectra, are therefore demanded. The combined HPLC–NMR technique has been developed during the last years. Its application has been expanded to several fields, including only very few examples of efficient identification of natural products from plants [2–4].

Because research on taxanes from Taxus species is widely performed, especially searching for new drugs based on Paclitaxel (Taxol) with promising

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anticancer activity [5], much more impetus has been given to the search for new taxanes from related plant species belonging to the family Taxaceae [6]. Thus, as a member of Taxaceae, the genus *Torreya* was emphasized. Several investigations from various points of view on *Torreya* plants have been reported and the results have been compared with those for *Taxus* species [7,8]. Furthermore, several pharmaceutically active components have been identified from plants in this genus [9,10].

Torreya jackii is found in a few groves scattered in southeastern China. In vivo experiments suggested its efficiency in reducing blood pressure and vasodilation [11–13]. Classical phytochemical investigation of this plant led to reports of the isolation of β-sitosterol and lignans [11–13]. In order to prove the applicability of the HPLC–NMR techniques for rapid phytochemical analysis we investigated *Torreya* needles by this method. To support the reliability of the evidence obtained from these HPLC–NMR spectra, additional phytochemical isolation of the corresponding components was performed, followed by ^{1}H-NMR measurements.

2. Experimental

2.1. Plant material

Fresh leaves were collected in Baishanzu National Park, Qiyang County, Zhejiang Province, southeastern China. Identified by Professor Zhongshu Yue, the voucher specimens are deposited at Kunming Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences. The collected foliage was air-dried (8.3 kg dry mass) and stored at 4°C for further treatment.

2.2. Sample preparation

0.8 g of a sample of dry needles of *T. jackii* was frozen by liquid N_2 and ground in a mortar. The powder was allowed to warm up to room temperature and was subsequently drenched in 2 ml MeOH before grinding vigorously. The slurry was transferred to a 15-ml Corex centrifuge tube. The mortar and pestle were washed twice with MeOH (2×2 ml) and the solutions were added to the Corex tube. The slurry was mixed by a Vortex for 10 min, frozen with liquid N_2 for 10 min, thawed and centrifuged at 14 400 rpm for 15 min. The supernatant was removed, diluted to 10-fold volume with double-distilled water and subjected to an Isolute C_{18} SPE (solid-phase extraction, Internationale Chemie-Technik, Bad Homburg, Germany) column (5 g/20 ml). The column was eluted successively with 35% MeOH (20 ml) and 55% MeOH (20 ml), and washed twice with 65% MeOH solution (2×20 ml). The collected solutions were combined and dried (1.2 mg). The residue was solved in 0.5 ml ice-cooled MeOH (HPLC grade, Merck, Darmstadt, Germany), and was mixed using a Vortex for 5 min at 4°C (with periodic placement in an ice bath). The clear solution was used for HPLC analyses and HPLC–NMR experiments.

2.3. Isolation

The air-dried leaves (8.3 kg) were exhaustively extracted by 2×40 l EtOH in a 55°C water bath. A dark-brown residue (1260 g) was obtained after evaporation in vacuum at 45°C. Thirty grams of the residue was defatted by 3×30 ml petroleum ether (60–90°C), followed by extraction with 3×40 ml chloroform. The chloroform extract (1.2 g) was subjected to silica gel column chromatography (80 g, 40–63 μm, Merck) using $CHCl_3$–MeOH (100:1–1:1) as gradient elution solvent. After further purification by prep. silica gel thin-layer chromatography (TLC) (0.5 mm, 60 F_{254}, Merck) six pure compounds were obtained: **1** (3.0 mg), **2** (3.2 mg), **3** (7.2 mg), **5** (4.8 mg), **6** (5.5) and **7** (5.6 mg). Compound **4** (1.2 mg) was obtained from the chloroform extract by reversed-phase HPLC on a LiChrospher 100 RP-18, 250×10 mm, 10 μm (Merck); UV 230 nm; flow-rate 3.0 ml min^{-1}; gradient from 25% to 40% MeCN in water (30 min).

2.4. Chromatographic and spectroscopic methods

HPLC–NMR spectra were obtained on a Bruker DRX 500 spectrometer at 500.13 MHz. A Merck-Hitachi LiChrograph L-6200A gradient pump was fitted with a 4 mm inverse-detection LC probe head (detection volume 120 μl). The chromatographic conditions described below were used in a stopped-

flow mode. Spectra were measured with a spectral width of 12 000 Hz, and 32 K data points were acquired. An acquisition time of 1.36 s and a relaxation delay of 1.80 s were used. Double solvent suppression of MeCN (Chromasolv, Riedel-de Haën, Germany) and HDO in the MeCN–^2H_2O (Deutero, Kastellaun, Germany) gradients were performed by presaturation applying standard Bruker pulse sequences. The suppressed signal of MeCN was set to δ 2.0 for calibration.

The following conditions were applied for HPLC: LiChrospher 100 RP-18, 250×4 mm; UV 230 nm; solvent A: MeCN+0.1% trifluoroacetic acid; solvent B: ^2H_2O+0.1% trifluoroacetic acid; flow-rate 0.8 ml min^{-1}; gradient: 0 min A–B (25:75), 30 min A–B (40:60), 40 min A–B (55:45). For a typical HPLC run ca. 300 μg of the pre-purified sample was dissolved in 20 μl MeOH-d_4 before injection.

NMR spectra of isolated compounds **1**–**3**, **5**, **6** and reference compounds were measured on a Bruker AMX 400 instrument at 400 MHz. Compounds **4** and **7** were measured on a Bruker DRX 500, at 500.13 MHz for ^1H and 125.7 MHz for ^{13}C. All spectra were recorded in acetone-d_6 using tetramethylsilane (TMS) as internal standard. EI mass spectra were performed at 70 eV on a Finnigan MAT 8230 and 44S spectrometer.

3. Results and discussion

The crude extract of only 0.8 g dry needles from *T. jackii* was sufficient for our investigations. As shown in Fig. 1 eight major components (**1–8**) were detected by HPLC–^1H NMR. From these peaks the ^1H NMR spectra were recorded by the stopped-flow method. Combining the results obtained by this way with those of EI-MS and CD spectra, the structures of seven of these compounds could be elucidated as follows.

Peak **1** represents a major compound in the extract. The HPLC–^1H NMR (Fig. 2) exhibited characteristics typical of a dibenzylbutyrolactone [14]. An AB quartet due to both H-7 benzylic methylene protons appeared with a large coupling constant of 13.9 Hz (Table 1). This pattern suggested that its neighbouring carbon was fully substituted. Further high field signals attributable to another benzylic methylene (H-7′α, H-7′β) and an aliphatic methine (H-8′) could be observed (Table 1). Further-

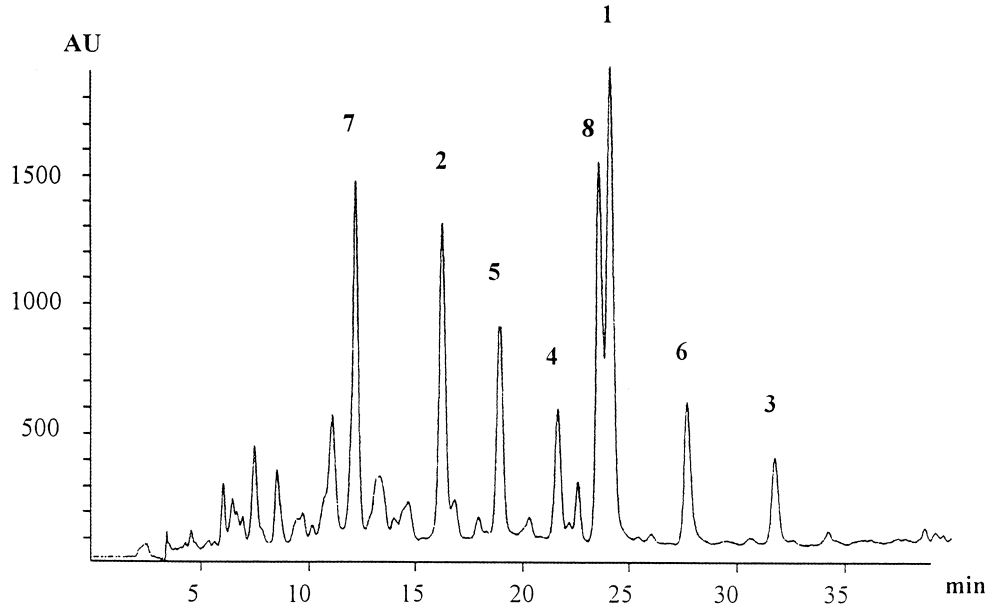

Fig. 1. HPLC chromatogram (UV 228 nm) of a pre-purified extract of *Torreya jackii* needles. Peaks were identified by HPLC–^1H NMR coupling as compounds **1**–**7**, respectively.

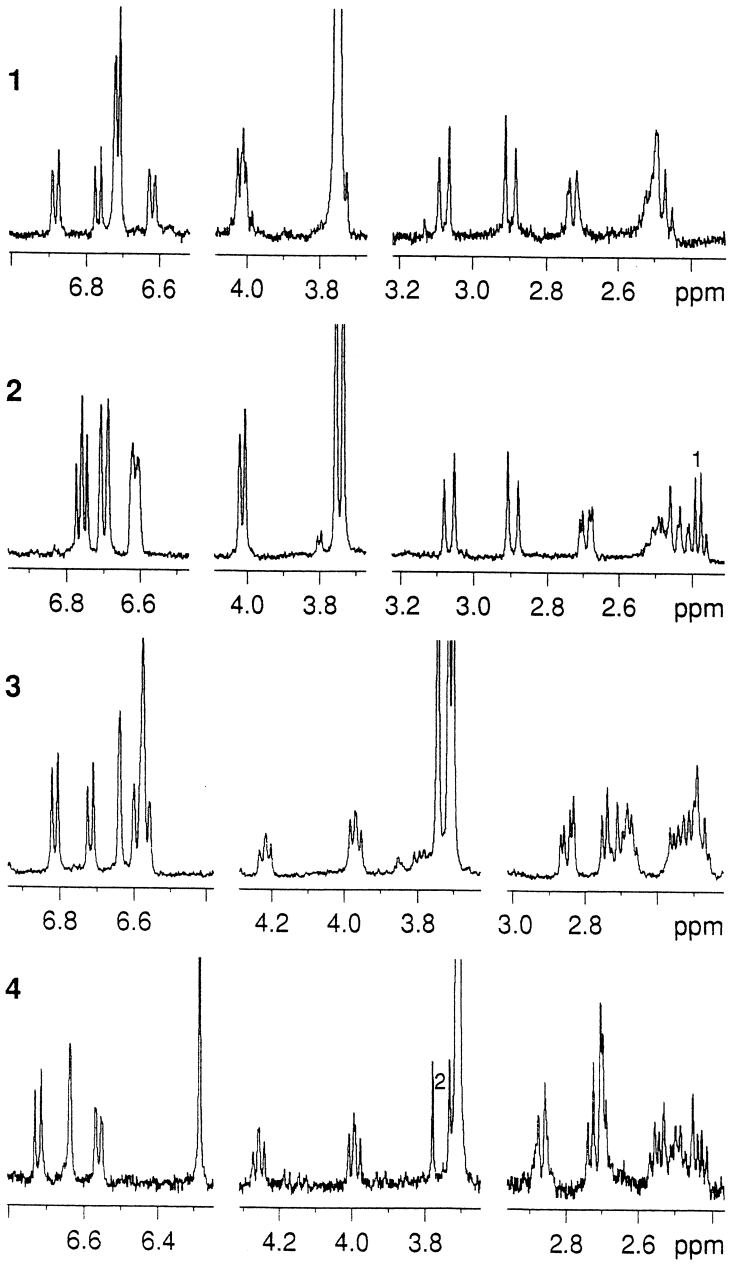

Fig. 2. HPLC–^{1}H NMR spectra of compounds **1**–**4**. Trachelogenin corresponds to peak **1** in the HPLC chromatogram; nor-trachelogenin= **2**; arctigenin=**3**; thujaplicatin methyl-ether=**4**. [1] Signal of EtCN, HPLC solvent impurity. [2] Signals of an impurity.

more, an oxygen-bearing methylene group exhibited two doublets close together indicating an 8-hydroxy-*trans*-8,8′-olide [14,15]. The aromatic part of the HPLC–^{1}H NMR spectrum exhibited signals of six protons belonging to two disubstituted rings. Combined with the fact that three aromatic methoxy groups could be observed, it could be deduced that one of the aromatic rings corresponds to a vanillyl group while the other is a veratryl moiety. This result suggested the structure was similar to that of the

Table 1
HPLC–^{1}H NMR spectral data of compounds **1**–**7** (500.13 MHz, MeCN–$^{2}H_2O$)

Comp.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Proton	δ, *J* (Hz)	δ, *J* (Hz)	δ, *J* (Hz)	δ, *J* (Hz)	δ, *J* (Hz)	δ, *J* (Hz)	δ, *J* (Hz)
2	6.70[a], s	6.69[a], s	6.58[a, i], s	6.64, brs, $\Delta_{1/2}$=4.4			6.96, s
2′	6.71[a], s	6.71[a], s	6.64[a], s	6.28[d], s			
5	6.76, d, 8.1	6.75[b], d, 8.1	6.72, d, 8.1	6.72, d, 8.1	6.83, s, 4H	6.83, s, 2H	6.75[p], s, 1H
5′	6.84, d, 8.1	6.77[b], d, 8.1	6.81, d, 8.1	–	6.95, s, 2H	6.94, m, 4H	6.79[p], s, 1H
6	6.61, d, 8.0	6.61[c], d, 8.1	6.57[b, k], d	6.56, dd, 8.1, 1.5			6.83[p], s, 2H
6′	6.71[f], d,	6.62[c], d, 8.1	6.59[b, k], d	6.28[d], s			
7α	2.89, d, 13.9	2.90, d, 13.9	2.72, dd, 13.9, 8.1	2.73, brd	4.73, d, 3.7, 2H	4.73[a], d, 3.7	–
7′α	2.48[g], m	2.45, d, 13.2	2.52[d], m	2.50[e, g], m		4.75[a], d, 3.7	2.58[d], t
7β	3.07, d, 13.9	3.07, d, 13.9	2.85, dd, 13.9, 5.1	2.87, brd	–	–	5.51, d, 6.6
7′β	2.72, brd, 9.5	2.69, dd, 13.2, 3.7	2.52[d], m	2.50[e, g], m	–	–	2.58[d], t
8	–	–	2.68, m	2.71, m	3.17, brs, $\Delta_{1/2}$=11.7	3.16, brs, $\Delta_{1/2}$=11.0	3.54[n], m
8′	2.48[h], m	2.49, m	2.49, m	2.50[l], m			1.77, m
9α	–	–	–	–	4.19, brdd, $\Delta_{1/2}$=18.3	4.19, brdd, $\Delta_{1/2}$=18.3	3.79[m], m
9′α	4.00[b], d, 7.3	4.01[d], d, 7.3	4.22, dd, 8.8, 7.3	4.26, dd, 8.8, 7.3			3.54[e, o], m
9β	–	–	–	–	3.80[d, m]	3.82[d, m]	3.79[m], m
9′β	4.01[b], d, 7.3	4.01[d], d, 7.3	3.97, dd, 8.8, 7.3	4.00, dd, 8.8, 7.3	3.80[d, m]	3.82[d, m]	3.54[e, o], t
MeO	3.75, s, 6H	3.74, s	3.70, s	3.70, s	3.81, s, 6H	3.78, s	3.78, s
	3.74, s	3.76, s	3.71, s	3.71, s (6H)		3.80, s	3.81, s
			3.74, s			3.81, s	

[a, b, c] May be reversed in one column.
[d, e] Common signals of equivalent protons in one column.
[f] Overlapped by H-2 and H-2′.
[g] Overlapped by H-8′.
[h] Overlapped by H-7′α.
[i] Overlapped by H-6 and H-6′.
[k] Overlapped by H-2.
[l] Overlapped by H-7′α/H-7′β.
[m] Overlapped by MeO signals.
[n] Overlapped by H-9′α/H-9′β.
[o] Overlapped by H-8.
[p] Signals of H-2′, H-5, H-6, H-6′.

known lignan trachelogenin [16]. To prove this assumption, compound **1** was isolated. The MS (M^+ *m*/*z* 388; $C_{21}H_{24}O_7$) and ^{1}H NMR data confirmed the constitution. The EI-MS fragment **13** (Fig. 3) indicated that the veratryl moiety is far from the carbonyl group in the olide ring, which is in agreement with the proposed structure. Moreover, nuclear overhauser effect (NOE) difference experiments (data not shown) were used to confirm the substitution pattern of the aromatic rings and provided also further evidence for the *trans*-fused olide ring. Stereochemically, the negative Cotton effect at 236 nm in the CD spectrum (data not shown) of **1** disclosed the presence of a 8*S*,8′*S* configuration (8α-OH), which had been clarified previously [16,17]. All these results are in complete agreement with the conclusion that component **1** is trachelogenin.

Peak **2** exhibited a very similar HPLC–^{1}H NMR spectrum as found for **1**, indicating the same structure of the *trans*-fused olide ring and both aromatic rings. However, the signals of H-5′ and H-6′ of compound **2** were shifted for about 0.1 ppm to higher field, indicating that the substitution pattern

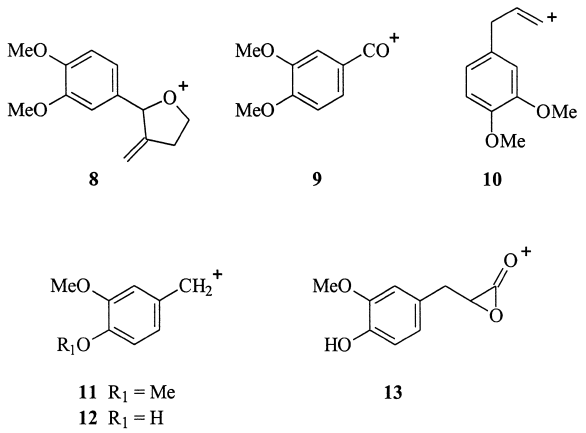

Fig. 3. Typical observed MS fragments from lignans of *Torreya jackii*.

has been changed in one of the phenyl rings. Together with the finding of only two methoxy signals the structure of that compound was in agreement with nor-trachelogenin [18].

The HPLC–^{1}H NMR spectrum of compound **3** (Fig. 2) showed some differences from those of **1**. Separated C-9′ protons (as double doublets) indicate an 8,8′-*trans*-oriented olide ring [19]. Moreover, the pair of doublets due to H-7α and H-7β in compound **1** was replaced in **3** by two further double doublets. This finding suggested the presence of a C-8 methine due to the signal at δ 2.68, which in part was superimposed with the signals of H-7′α and H-7′β appearing as a multiplet centered at δ 2.52 (Fig. 2). The signal at δ 2.49 was attributed to H-8′. As the number of methoxy signals was three, the aromatic rings were assumed to be a veratryl and a vanillyl unit.

The significant fragments **10**, **11** and **12** (Fig. 3) in the mass spectrum of the isolated compound **3** confirmed both units and the fragment **13** indicated that the veratryl unit is located far from the carbonyl site [14]. A negative Cotton effect at 234 nm, was in agreement with the 8*S*,8′*R* configuration [19]. All data were consistent with compound **3** being due to (−)-arctigenin [20,21].

The aliphatic signals in the HPLC–^{1}H NMR spectrum (Fig. 2) of peak **4** indicated the same lignan type as (−)-arctigenin (**3**). However, only five aromatic protons were detectable, three of them owing to a vanillyl unit. Other NMR data pointed to a triply substituted phenyl moiety and the occurrence of three methoxy signals in this spectrum accounted for one vanillyl unit and a 4-hydroxy-3,5-dimethoxy substituted ring. NMR spectral data (HMBC, data not shown) of the isolated compound, were used to assign the position of the 4-hydroxy-3,5-dimethoxy-phenyl ring close to the carbonyl group. Thus, **4** must be thujaplicatin methyl-ether [22]. The mass spectrum clearly confirmed this finding.

The HPLC–^{1}H NMR spectrum of peak **5** showed only six groups of signals (Fig. 4). Two groups of aromatic hydrogens, two signals for H-7α/7′α and H-9α/9′α, a singlet due to two methoxy groups in part overlapped by the signal attributed to H-9β/9′β and a singlet assigned to H-8/8′ could be observed. This spectrum strongly suggested that peak **5** was due to a symmetrical dibenzylfurofuran lignan, such as pinoresinol [14,23]. Literature data [24] and HPLC–^{1}H NMR data of authentic pinoresinol identified **5** unambiguously as pinoresinol (Fig. 5).

Peak **6** exhibited an HPLC–^{1}H NMR spectrum (Fig. 4) very similar to that of **5**. Comparison of these two spectra indicated three points of difference. First, two benzylic methylene signals attributable to H-7α and H-7′α appeared separately at δ 4.73 and δ 4.75. Second, the aromatic signals indicated that the two aromatic rings of **6** are different. Finally, three methoxy singlets appeared in the HPLC–^{1}H NMR spectrum of **6,** which is therefore possibly a mono-methyl ether of **5**. This assumption was confirmed by MS and NMR data of isolated **6**. The molecular ion of **6** at *m*/*z* 372 corresponds to a molecular formula of $C_{21}H_{24}O_6$ and the diagnostic mass fragments **8** and **9** (Fig. 3), pointed to the presence of a vanillyl and a veratryl unit in this structure. The relative position of the sole OH group at C-4′ was deduced from NOE difference measurements. By comparing the conventional ^{1}H NMR spectral data of **6** (not shown) with those reported in the literature [25], the proposed structure of **6** was confirmed as methylpinoresinol.

The HPLC–^{1}H NMR spectrum of component **7** was unlike to those of the spectra of **1–6**. Signals of five aromatic protons, a doublet of an oxygen substituted methine proton, signals of seven aliphatic protons and another multiplet at δ 3.79 (Table 1) indicated a neolignan type compound but, in contrast to the products **1–6**, did not allow direct identifica-

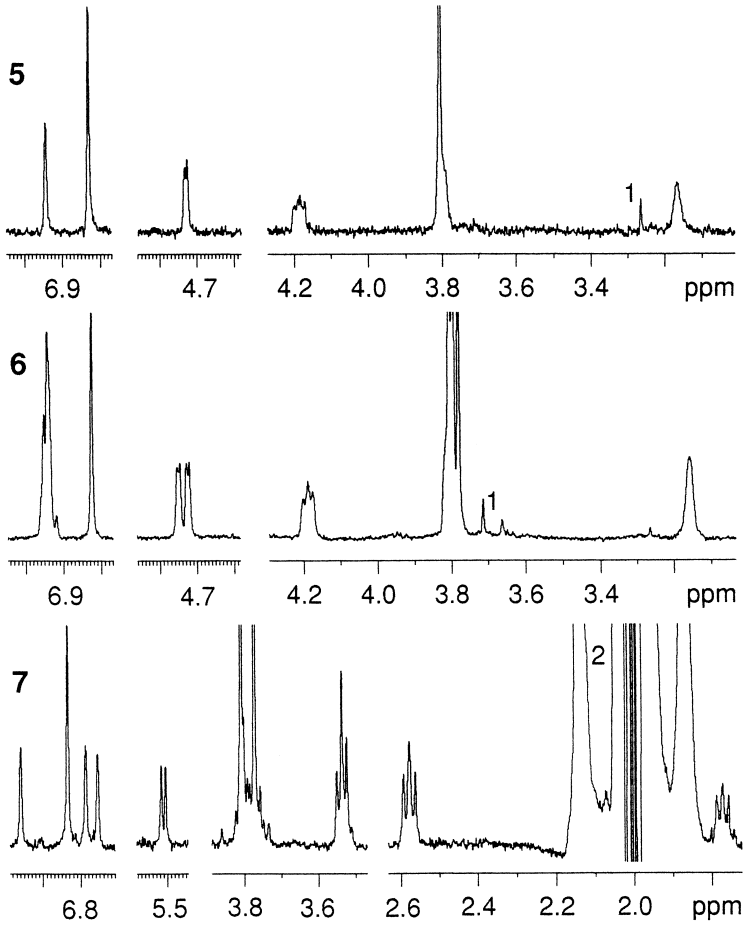

Fig. 4. HPLC–^{1}H NMR spectra of compounds **5**–**7**. Pinoresinol corresponds to peak **5** in the HPLC chromatogram; methylpinoresinol=**6**; dihydrodehydrodiconiferylalcohol=**7**. [1] Signals of an impurity. [2] Large signals of MeCN and its satellites used as HPLC solvent.

tion from the HPLC–NMR data in that case. Isolation and conventional analysis by ^{1}H NMR, ^{1}H-^{1}H COSY, inverse detected one-bond and multiple-bond ^{1}H-^{13}C correlated two-dimensional (2D) spectra (HMQC and HMBC) revealed the structure of compound **7** being dihydrodehydrodiconiferylalcohol and also allowed assignment of signals on the HPLC–^{1}H NMR spectrum. Component **8** was also measured by HPLC–NMR. It is a non-lignan type compound of currently unknown structure.

4. Conclusions

In conclusion, it could be clearly demonstrated that stopped-flow HPLC–^{1}H NMR represents a powerful analytical technique for structure elucidation of natural products in crude extracts from plant material. The entire procedure described here is very rapid. It involves only 2 h of sample pre-purification, a preliminary HPLC run to check chromatographic separation, and 30 min, on average, for running each of the HPLC–^{1}H NMR spectra in the stopped-flow mode. Seven lignans were identified by HPLC–^{1}H NMR, and, if necessary, the proposed structures were confirmed later by conventional ^{1}H NMR and MS spectra of the isolated compounds and reference samples. From our experience it is obvious that MS data are indispensable for a final structure determination. After HPLC–NMR measurement, the sepa-

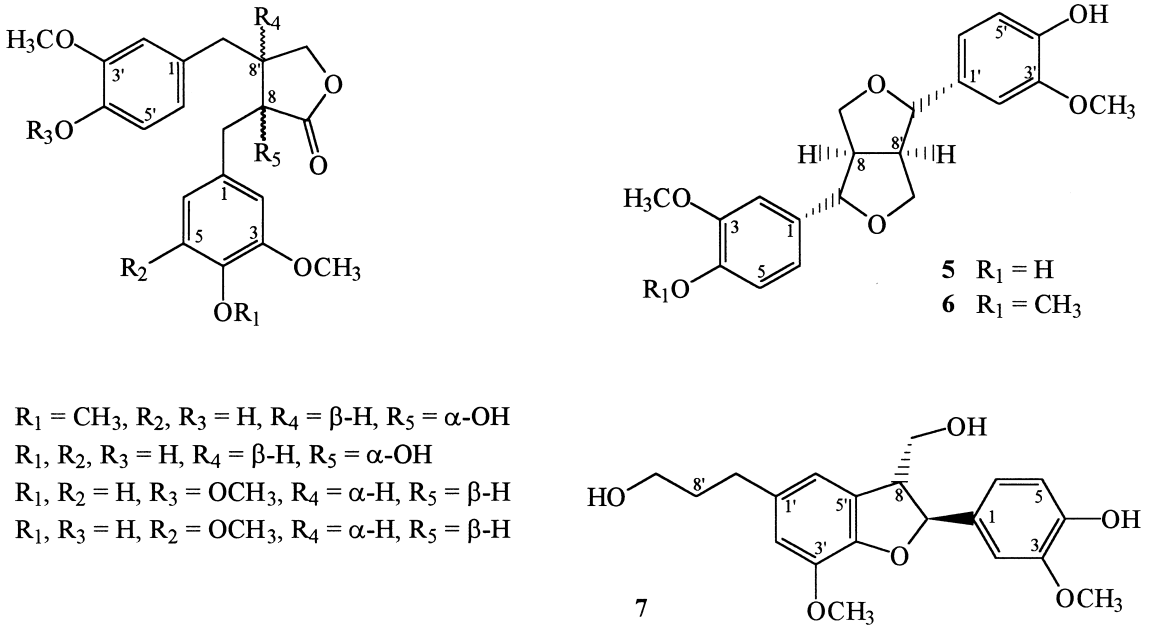

Fig. 5. Chemical formulas of identified lignans from *Torreya jackii* needles.

rated samples were collected and used for running mass spectra. In the near future, the application of routine HPLC–MS–NMR systems for automatic screening of natural products therefore is highly desirable and would be in combination with high throughput screening (HTS) systems a most efficient technique to detect new compounds with interesting pharmacological activities.

5. Author query

Please provide a reference in the body of Table 1 for footnote h.

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