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Full-length paper

HPLC-coupled spectroscopic techniques (UV, MS, NMR) for the structure elucidation of phthalides in *Ligusticum chuanxiong*

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Summary

Ligusticum chuanxiong Hort., a plant that is frequently used in traditional Chinese medicine, has been studied using HPLC-coupled spectroscopic techniques such as HPLC-UV, HPLC-MS as well as HPLC-NMR. With the aid of these modern spectroscopic techniques, the main constituents, namely senkyunolide A, butylphthalide, neocnidilide and *Z*-ligustilide, have been characterized and identified. Phthalide dimers, present in smaller amounts, have been identified by HPLC-UV and HPLC-MS analysis and compared with reference compounds. Stereochemical features of some phthalide monomers have been determined by detailed spectroscopic studies for the first time.

Abbreviations: APCI-MS, atmospheric pressure chemical ionization mass spectrometry; DPFGNOESY, double pulsed field gradient enhanced nuclear Overhauser spectroscopy; GC-MS, gas chromatography-mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; HPLC-DAD, high performance liquid chromatography with diode array detection; HPLC-MS, high performance liquid chromatography with mass spectrometry detection; HPLC-NMR, high performance liquid chromatography with nuclear magnetic resonance detection; TLC, thin layer chromatography; TOCSY, total correlation spectroscopy; TCM, traditional Chinese medicine; UV, ultraviolet

Introduction

Traditional Chinese Medicine (TCM) is predominantly based on acupuncture, moxibustion and the use of drugs, mainly of plant origin. The total number of Chinese plants used for medicinal purposes is estimated to be about 5000 [1]. Many of them have been used to treat rheumatism, migraine, asthma and allergies. Quality control of drugs seems to be an inevitable starting point for a safer use of these drugs in medicinal applications. Different drugs can carry the same name, for instance "*Pien Hsu*" refers to a drug made from *Polygonum aviculare* in China, whereas in Taiwan it would be *Euphorbia thymifolia* [2]. Using one or the other can have dramatic consequences and lead to severe poisoning [3]. Moreover, the quality control of Chinese drugs demands rapid, easy and simple analysis. HPLC determination of the constituents of mixtures is a well-established tool, provided that the identification of the components has been verified by spectroscopic techniques. When searching for the active principles of these drugs, the application of on-line spectroscopic techniques, such as HPLC-MS and HPLC-NMR, is an alternative in order to identify known compounds without isolation and replication. In recent years hyphenated HPLC techniques focusing on structure elucidation like HPLC-MS and HPLC-NMR have become widely available [4, 5] and numerous applications with either one of these techniques or combinations of them have been published in literature. It should be noted at this point that only NMR studies provide detailed stereochemical information of the compounds under investigation. Hence, HPLC-NMR investigations have been applied to the analysis of the roots of *Ligusticum chuanxiong* Hort., a well-known drug in traditional Chinese medicine frequently used for the treatment of gynecological disorders, headache and rheumatism.

In earlier investigations, which were carried out with the Japanese substitute for *L. chuanxiong*, the very closely related species *Cnidium officinale*, structure elucidation of the main constituents, namely phthalides, was based on

chemical characterization of the isolated compounds and subsequent comparison of spectra [6–10]. Recently HPLC-MS investigations have been reported for *L. chuanxiong* [11, 12].

Results and discussion

The *n*-hexane extract of *Ligusticum chuanxiong* could be clearly separated by reversed phase HPLC analysis [14]. Peak 1 (Rt = 8.6 min) gave a simple UV-spectrum with only one broad maximum at 280 nm. APCI-MS analysis of peak 1 resulted in a molecular weight of *m/z* 193 ($[M+H]^+$), suggesting that the underlying compound could be the monomeric phthalide senkyunolide A **1** found in this plant earlier [15, 16]. This finding was confirmed by the corresponding ^{1}H-NMR spectrum of Peak 1, obtained by HPLC-NMR analysis (Figure 1). In the ^{1}H-NMR spectrum two olefinic protons at $\delta = 5.97$ ppm (dt) and $\delta = 6.07$ ppm (dt) were observed. A further proton at $\delta = 5.05$ ppm (dd) was assigned to be H-3. By selective TOCSY experiments with $\delta = 5.97$ ppm as the irradiation point, the spin-system H-7, H-6, H-5, H-4 became visible. With these experiments protons at position H-5 ($\delta = 2.4$ ppm) and H-4 ($\delta = 2.5$ ppm) could be assigned. In the same manner irradiation at position $\delta = 5.05$ ppm visualized the spin-system H-3, H-8 (H-8b: $\delta = 1.57$ ppm, H-8a obscured by acetonitrile), H-9 ($\delta = 1.22$–1.34 ppm), 10-H ($\delta = 1.22$–1.34 ppm), 11-H ($\delta = 0.84$ ppm). These data proved unambiguously that the underlying compound of peak 1 was senkyunolide A.

HPLC-peak 2 (Rt = 9.2 min) was investigated by the same protocol. The UV spectrum of this peak showed three maxima at 203, 230 and 275 nm, indicating a change in the chromophore in comparison to senkyunolide A. HPLC-MS analysis using APCI-MS indicated with $m/z = 191$ for $[M+H]^+$, a molecular weight with only two protons fewer than senkyunolide A. This finding was corroborated by the HPLC-NMR spectrum (Figure 1), where two olefinic protons of an additional double bond were found, which could be most obviously described as aromatic protons. Chemical shifts as well as coupling patterns (see Experimental) indicated a disubstituted aromatic compound. In addition, the signals for the butyl sidechain were identified as H-3 ($\delta = 5.58$ ppm), H-8 (obscured by acetonitrile), H-9 ($\delta = 1.2$–1.4 ppm), H-10 ($\delta = 1.2$–1.4 ppm), and H-11 ($\delta = 0.84$ ppm). Thus, butylphthalide **2**, a compound also described earlier for this plant [17], was identified.

HPLC-peak 3 at Rt = 11.8 min displayed only one single olefinic peak at $\delta = 6.77$ ppm in the ^{1}H-NMR-spectrum (Figure 1). Also in this case HPLC-MS analysis provided the information about the molecular weight of the compound. The molecular peak with $m/z = 195$ ($[M+H]^+$) confirmed the presence of another monomeric phthalide, this time with one fewer double bond than senkyunolide A. The ester function was identified by a resonance at $\delta = 4.2$ ppm. Careful 1D-selective TOCSY experiments identified the spin system H-7, H-6, H-5, H-4 by selective excitation of proton H-7, as well as the butyl side chain by selective excitation of the terminal methyl group or the ester function at position H-3.

In order to obtain stereochemical information about the ring-junction and the relative stereochemistry of the side chain, the two possible isomers were modeled using PCMODEL (MMX-force-field, Serena Software). These calculations (Figure 2) indicated, that for the structure of neocnidilide **3**, which was suggested by Mitsuhashi and Muramatsu [6] isolated from *Cnidium officinale*, a nuclear Overhauser enhancement should be possible for H-5_a as well as for H-4_a upon irradiation of proton H-3. Unfortunately H-4 was obscured by the solvent signal. Nevertheless a clear N.O.E could be observed between H-5 and H-3 and vice versa by using DPFGNOESY experiments when doing selective excitation of H-5 and H-3 respectively (Figure 3). Thus, for the first time the stereochemistry of neocnidilide was confirmed by NMR. Previously this was proven only by synthesis [9, 10].

Peak 4 (Rt = 12.2 min) was identified by HPLC-MS analysis as a monomeric phthalide with the molecular weight 190 (m/z $[M+1]^+ = 191$). ^{1}H-NMR (see Experimental), 1D-selective TOCSY as well as MS results confirmed peak 4 to be *Z*-ligustilide **4**. The UV-spectrum with maxima at very long wavelengths (λ_{max} (nm) $\nu = 209$, 242, 252, 282, 294 and 327) can be explained by the complex chromophore of *Z*-ligustilide, where three conjugated double bonds are in cross conjugation to the lactone ring. *Z*-ligustilide was found to be a major component in the volatile oils of several plants from the Apiaceae family, e.g. in *Angelica sinensis* [18], *Levisticum officinale* [6, 19] and *Cnidium officinale* [6].

In addition to the monomeric phthalides, the occurrence of the dimeric phthalides tokinolide B **5** (Rt = 21.0 min) and levistolide A **6** (Rt = 21.9 min) was supported by HPLC-MS studies showing molecular ion peaks at *m/z* at 381 and 383, respectively. This was confirmed by direct comparison with reference compounds tokinolide B and levistolide A from *L. chuanxiong* [16].

Furthermore, butylidenephthalide **7** (Rt = 12.6 min) and falcarindiol **8** (Rt = 17.1 min) were identified by direct comparison with reference substances isolated from *Angelica sinensis* [12] and from *Notopterygium incisum* [21], respectively. For HPLC-MS as well as for HPLC-NMR analysis the concentration of these compounds in the extract was not sufficient.

An interesting feature in the HPLC-MS-analysis of *L. chuanxiong* was the observation that dimeric phthalides were more easily protonated by APCI-MS than the respective monomeric phthalides. This can be seen from the high signal intensities of the phthalide dimers and from the observation that the main peak found in the mass spectra was $[M+H]^+$ for phthalide dimers, but $[M+H+CH_3CN]^+$ for phthalide monomers. This is of practical importance due to the fact that phthalide dimers cannot be analyzed by GC-MS because of a *retro*-Diels-Alder reaction occurring already below 100 °C. [21]. The analysis of monomeric phthalides, especially that of *Z*-ligustilide, was also reported to be problematic because

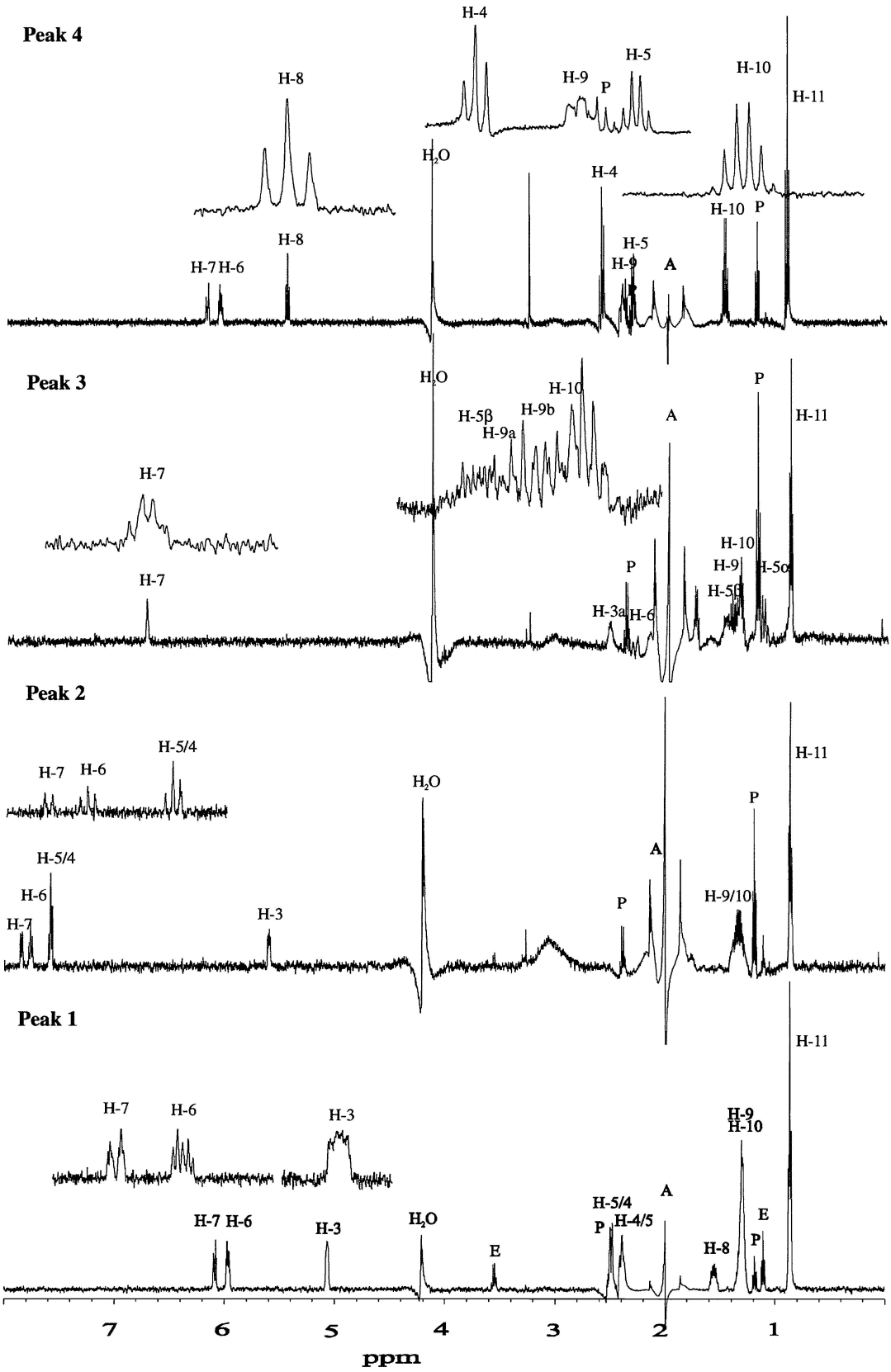

Figure 1. HPLC-NMR of compounds **1**-**4.** Shown are the ^{1}H-NMR spectra under stop-flow conditions. HPLC, and NMR conditions, see experimental.

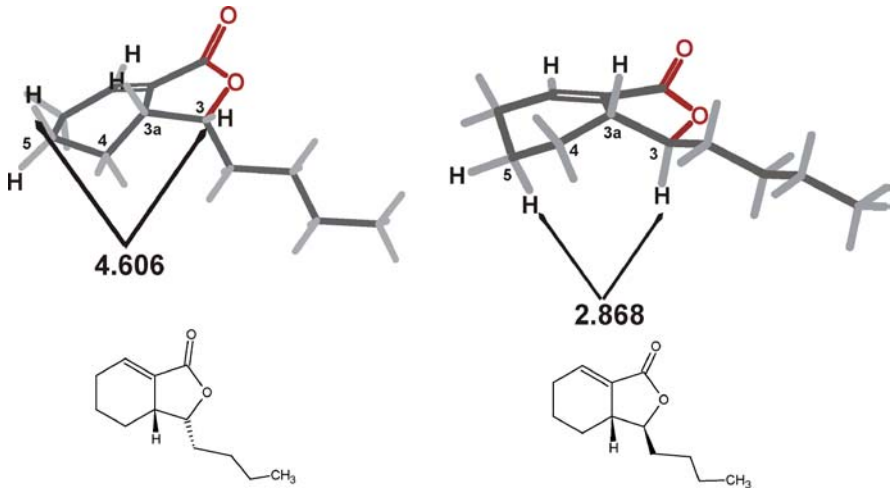

Figure 2. Calculated structures for neocnidilide **3** using PCMODEL (Serena Software) and MMX as force-field.

of the generation of isomeric products during GC-analysis [22].

In conclusion, the presence of senkyunolide A, butylphthalide, neocnidilide and *Z*-ligustilide in the *n*-hexane extract of *L. chuanxiong* was confirmed by the combination of HPLC-DAD, HPLC-MS as well as HPLC-NMR analytical techniques. These compounds represent the main constituents of the nonpolar extract of this plant. Having confirmed their structures, HPLC-DAD analysis can be recommended as an excellent tool for the analysis of this frequently used medicinal plant. The obtained UV-spectra for peaks 1–4 are very characteristic due to the different chromophores found in the monomeric phthalides. The chromophores range from a single double bond in conjugation with a lactone group in the case of neocnidilide **3** resulting in a simple UV-spectrum with only one maximum at 222 nm, to the aromatic constituent butylidenephthalide **7** (peak 5), in which the benzene ring, the lactone ring and an additional conjugated double bond lead to a very complex UV-spectrum with maxima found at long wavelengths (λ_{max} (nm) $\nu = 211, 218, 238, 260, 272, 312$).

In addition it has been shown that with the aid of HPLC-coupled spectroscopic methods even compounds in μg quantities can be directly assigned without isolation. The strength of the HPLC-NMR investigation seems to be the possibility for stereochemical investigations.

Experimental

Samples

Five samples of *L. chuanxiong* Hort., roots from different sources, have been analyzed: TCM-Hospital, Kötzting, May 1996 [**1**]; TCM-Hospital, Kötzting, July 1996 [**2**]; Oriental Medicine Research Center of the Kitasato Institute, Japan[**3**]; pharmacy**,** Singapore [**4**]; drug market, Shenyang [**5**]. After proving conformity of the different samples by TLC and HPLC analysis, HPLC-MS and HPLC-NMR experiments were carried out with sample **1**.

Extraction

Five grams powdered plant material were extracted under reflux for 2 h with 50 ml of *n*-hexane; the extracts were evaporated to dryness, weighed and redissolved in ethanol p.a.; the concentration for HPLC-UV analysis was 10 mg/ml, for HPLC-MS and 50 mg/ml HPLC-NMR. The samples were filtered through an HPLC membrane filter (0.45 μm, Roth, Art 5992.1) prior to injection.

HPLC-DAD

Instrumentation: Hewlett-Packard HP 1050 liquid chromatograph equipped with an HP 1040 M photodiode-array detector. Data analysis was carried out on HP Chemstation. Column: LiChroCART® 125-4 with LiChroSpher® 100 RP-18 (5 μm), Merck; precolumn: LiChroCART® 4-4 with LiChroSpher® 100 RP-18 (5 μm), Merck.

Mobile phase: A = distilled water, B = acetonitrile (J. T. Baker, HPLC Ultra Gradient Grade); gradient: 40–55% B, linear in 15 min, 55–95% B, linear in 18 min, 95% B, isocratic for 2 min; flow: 1 ml/min; injection volume: 10 μl; column temperature: 40 °C; UV-detection: 210 nm.

HPLC-MS

HPLC Instrumentation**:** Liquid chromatograph ABI, pump 140B, UV-detector 785A (Applied Biosystems). HPLC/APCI-experiments were performed with a mass spectrometer Finnigan TSQ 700 (Finnigan MAT); temperature of the vaporizer: 350–450 °C; corona current: 5 mA;

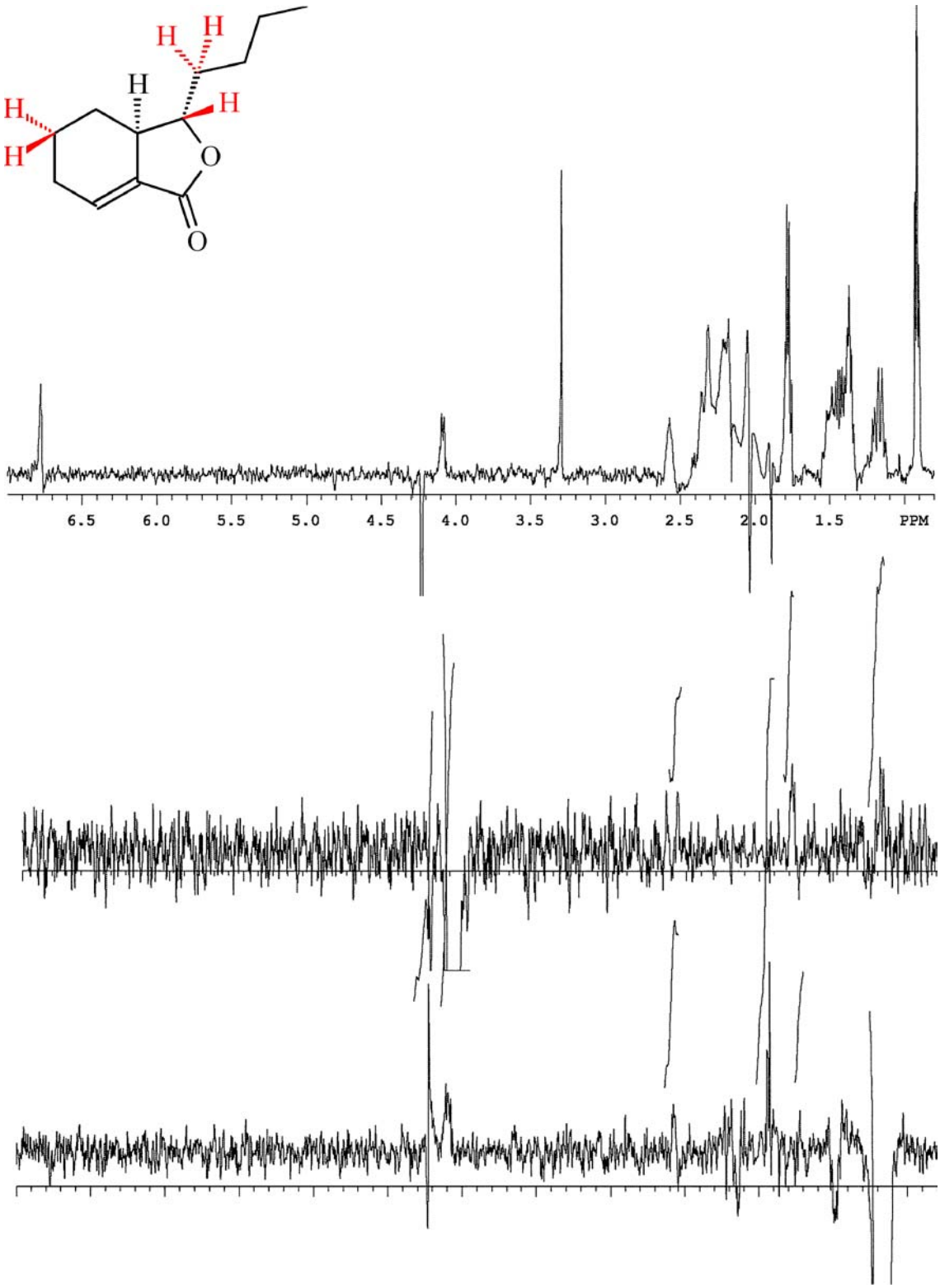

Figure 3. NOE measured by DPFGNOESY experiments. The upper trace shows the regular ^{1}H-NMR. Lower two traces show selective excitation of H-5 and H-3, respectively.

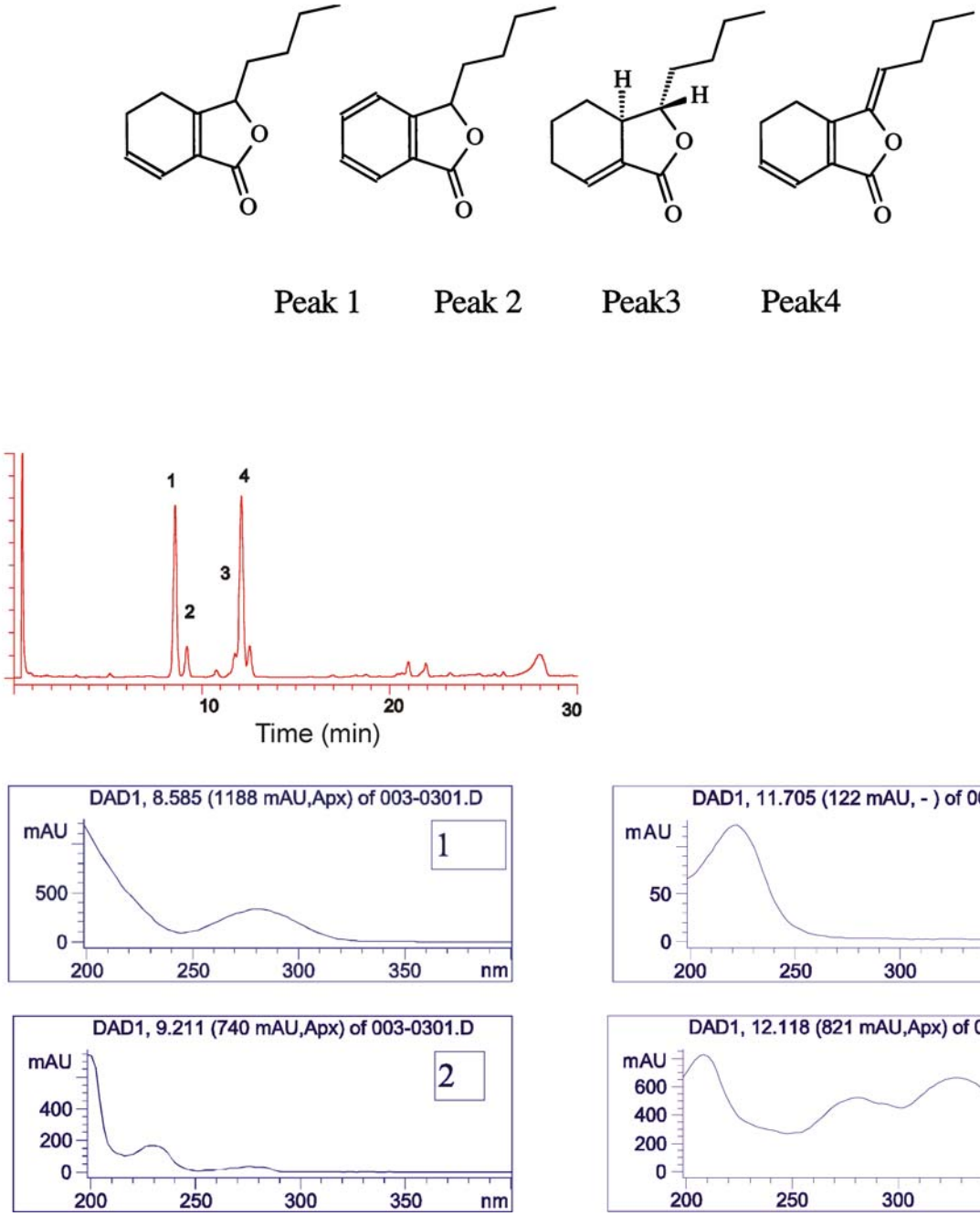

Figure 4. HPLC-chromatogram (trace taken at $\lambda = 235$nm), and diodearray UV-spectra for compounds **1**–**4**.

temperature of heated capillary: 200 °C: Column and gradient: see HPLC-UV.

HPLC-NMR

HPLC-Instrumentation: Varian liquid chromatograph with gradient pump 9012 and 9050 UV-detector. NMR-spectrometer: Varian Unity INOVA 500 MHz probe: PFG-HPLC-probe (Varian); flow cell with 60 μl active volume. Column: YMC-column 250 mm × 3 mm, J'sphereM 80/80 A (3–4 μm). Mobile phase: A = D_2O, B = acetonitrile. Gradient: 25–50% B linear in 25 min, 50–95% B linear in 10 min, isocratic for 5 min. Flow: 0.8 ml/min; detection: 210 nm; injection volume: 50 μl. All spectra were taken under stop-flow conditions by stopping the HPLC run at the appropriate peak and than performing the necessary NMR-experiments. 1D-TOCSY as well as DPFGNOESY [13] spectra were taken from the standard Varian pulse library. Mixing time for TOCSY was varied from 120 ms to >5 ms to see the whole or parts of the spin system. Mixing time for DPFG-NOESY was 1 sec, repetition time 3 sec. All selective pulses were created using Pbox.

Senkyunolide A 1 (peak 1)

UV λ_{max} (nm) 280. LC-^{1}H-NMR(500 MHz) δ 6.07 (1H, dt, 9.8, 1.0 Hz, H-7), 5.97 (1H, dt, 9.8, 4.4 Hz, H-6), 5.05 (1H, dd, 7.0, 3.0 Hz, H-3), 2.5 (2H, m, H-4), 2.4 (2H, m, H-5), 1.57 (1H, m, H-8_b), 1.22–1.34 (4H, m, H-9, H-10), 0.84 (3H, t, 7.2 Hz, H-11). Signal H-8_a under solvent-signal (acetonitrile).

Butylphthalide 2 (peak 2)

UV λ_{max} (nm) 203, 230, 275. LC-^{1}H-NMR(500 MHz) δ 7.8 (1H, d, 7.6 Hz, H-7), 7.75 (1H, t, 7.6 Hz, H-6), 7.55 (1H, d, 7.5 Hz, H-4), 7.55 (1H, t, 7.5 Hz, H-5), 5.58 (1H, dd, 8.0, 3.5 Hz, H-3), 1.2–1.4 (4H, m, H-9, H-10), 0.85 (3H, t, 7.0 Hz, H-11). Signals H-8_a and H-8_b under solvent-signal (acetonitrile).

Neocnidilide 3 (peak 3)

UV λ_{max} (nm) 222. LC-^{1}H-NMR (500 MHz) δ 6.77 (1H, q, 3.5 Hz, H-7), 4.2 (1H, m, H-3), 2.53 (1H, m, H-3a), 2.33 (1H, m, H-6a), 2.2 (1H, m, H-6b), 1.7-1.79 (2H, m, H-8), 1.45-1.55 (1H, m, H-5a), 1.4-1.45 (2H, m, H-9), 1.3-1.4 (2H, m, H-10), 1.15 (1H, m, H-5b), 0.9 (3H, t, 7.3 Hz, H-11). Signals H-4a, H-4b under solvent-signal (acetonitrile).

Z-Ligustilide 4 (peak 4)

UV λ_{max} (nm) 209, 242, 252, 282, 294, 327. LC-^{1}H-NMR (500 MHz) δ 6.21 (1H, dt, 9.5, 1.5 Hz, H-7), 6.1 (1H, dt, 9.5, 4.2 Hz, H-6), 5.5 (1H, t, 8.0 Hz, H-8), 2.61 (2H, t, 9.7 Hz, H-4), 2.45 (2H, m, H-9), 2.4 (2H, m, H-5), 1.5 (2H, qt, 7.3, 7.3 Hz, H-10), 0.94 (3H, t, 7.3 Hz, H-11).

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