

Liquid chromatography-mass Spectrometry Dereplication Strategy for Isolation of Oligostilbenes

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Abstract

The leaves of *Neobalanocarpus heimii* were investigated for their oligostilbene contents. Prior to isolation process, the determinations of compounds were based on mass spectrometric fragmentation patterns. Three compounds, heimiol B, hopeaphenol and vaticaphenol A were identified directly from the crude extract. Preparative high performance liquid chromatography (HPLC) was used to isolate and purify the other compounds. The purified compounds were then analyzed using NMR spectroscopy to identify the compound structure and stereochemistry. The result shows that the leaves extract of *N. heimii* contain three other oligostilbenes; vaticanol A, balanocarpol and vaticaphenol A, and a galactopyranose.

Keywords: Balanocarpol, Hemiol B, Hopeaphenol, Vaticanol A, Vaticaphenol A.

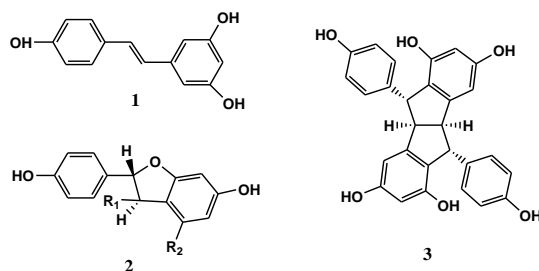
INTRODUCTION

Neobalanocarpus heimii (King) Ashton locally known as cengal is endemic to Southern Thailand and Peninsular Malaysia. It is an important genus as it produces wood that has become the standard by which many others are compared. It is fairly widely distributed but found in scattered occurrence and at low numbers.¹The area of natural distribution is limited to Peninsular Malaysia, Thailand and Indonesia where it may be extinct. In Malaysia it has been one of the most popular hardwoods and has been heavily logged throughout the state. It is found in tropical lowland forests below 1000 m altitude, especially on well-drained soils on undulating land. In Thailand it occurs in hilly dipterocarp forest along slopes and in valleys. Best growth is achieved

in areas with more than 2000 mm rain per year and no prolonged dry season. It is not tolerant to frost.²

In recent years, stilbenes, which exist in natural kingdom, have attracted much attention for their various biological activities, including antimicrobial, anti-cancer, anti-inflammation, hepatoprotective and hepatotoxic activities.³ From 1995 to 2008, about 100 out of more than 400 stilbene derivatives reported were isolated from Dipterocarpaceae plants.⁴ Plants from the Dipterocarpaceae family have proven to be a rich source of oligostilbene compounds derived from a stilbene, resveratrol (4,3,5'-trihydroxystilbene).⁵

Resveratrol (**1**) is a stilbenoid, a natural phenol and a phytoalexin produced by several plants when the plant is under attack. Oligomer stilbenoids can be classified into two groups which first, contain at least one 5-membered oxygen heterocyclic ring, usually the trans-2-aryl-2,3-dihydrobenzofuran moiety (**2**) and the second group does not contain any oxygen heterocyclic ring, such as pallidol (**3**).⁵



EXPERIMENTAL

Procedure for dereplication strategy:

Optimization of LC-MS System for Database Building

In this study, crude extracts from dipterocarp plant was used as model samples. Mass spectrometric data of eleven pure compounds were recorded for a database. LC-MS system was optimized in order to obtain MSⁿ data of each pure compound. This optimization comprises ionization and fragmentation parameters. MSⁿ data of compound were recorded and stored in ion trap LC-MS system as a library of compound data to be used as reference data.

Optimization of Chromatographic Conditions

The chromatographic conditions were obtained on a UPLC system. This includes choice of the chromatographic column, temperature, solvent system, gradient profile and flow rate of delivery solvent. The optimized chromatographic conditions were used in the LC-MS system.

Identification of Plant Constituents by Ion Trap LC-MS

The automatic identification system involves an ion trap system and a database. MSⁿ data of biomarker compounds in the crude extract were compared with those stored in the system as library of compounds data.

Procedure for Isolation of Compounds:***Extraction of Plant Material***

A classical extraction technique, which is maceration, was used for the extraction of *N. heimii* leaves. The technique is based on the extracting power of different solvents in use and the application of mixing. The plant material was ground into small particle. In maceration process, a mixture of water and acetone (1:1) was used and the sample was left macerated overnight. Later the extract was strained off and the extraction solvent was removed to obtain a crude residue. The process was repeated until the test for phenolic presence was negative.

Optimization of Chromatographic Conditions

The chromatographic conditions were obtained on a UPLC system. This includes choice of the chromatographic column, temperature, solvent system, gradient profile and flow rate of delivery solvent. The optimized chromatographic conditions were used in the preparative HPLC for isolation and purification purposes. Apart from the chromatographic column, very fraction has a specific chromatographic condition.

Isolation and Purification of Oligostilbenes

The crude drug was injected into preparative HPLC using individually optimized chromatographic conditions. Each sample was prepared as 450 mg in 10 ml methanol. The separation was carried out over a Supelco (5 μ m, 20 x 150 mm) semi preparative column. The detection was at wavelength 215nm and 283nm. The process was run repetitively until enough samples were obtained for further purification. The purification procedure was similar as of the isolation, but using smaller column ID. The process was repeated until enough only one peak observed in the chromatogram indicating pure compound.

Procedure for compound identification:

The purified sample was dried using rotary evaporator. The sample was dissolved in deuterated acetone and transferred into a NMR tube. The NMR data were recorded for each sample.

RESULTS AND DISCUSSION***Dereplication Strategy for Compound Identification***

For the rapid identification of the oligostilbenes from the leaves of *N. heimii*, the methanolic crude extract was directly injected into the ion trap LC-MS system. A 1-mg/ml sample was prepared in acetonitrile and was analyzed in different experiments. The chromatographic profile of Total Ion Current (TIC) of the crude extract was obtained. The retention times were different with those of HPLC analysis. Both analyses were performed under different chromatographic conditions, where the column and flow rate were adjusted to suit the instrument requirements and for better resolution. Altogether, the retention times were different but the sequence of elution seemed to be identical.

The intensity of the peaks in both chromatograms is also different. This can be explained by considering the difference in the detection method for both UV and MS techniques. The UV detector measures the molar absorptivity whereas the MS detector measures the ionizability of the compounds. The response might be quite different for the same compound when detected by UV or MS.

The TIC of the LC-MS analysis provides on-line molecular mass information. This helps in estimating the oligomerization degrees of the stilbenes. As the resveratrol (the biogenetic precursor of all oligostilbenes isolated in this work) mass is 228, a dimeric stilbene mass would be around 450-480, considering inter-monomer bonding and the possibility of excess oxygen atoms. A trimeric stilbene mass would be around 680-700 and for a tetrameric stilbene, around 900-950.

The analyses were continued with MS², MS³ and so on until there are no more ions to be fragmented. The fragmentation patterns extracted from the experiments were compared with those of the pure compounds isolated previously. The fragmentation patterns are unique for each compounds regardless the retention times and intensity of their peak. This will ensure positive identification directly from a mixture. After considering the co-elutions and overlapping peaks, five compounds were positively identified. Figure 1 shows the chromatogram of the crude extract and the identified peaks correspond to the compounds from the library.

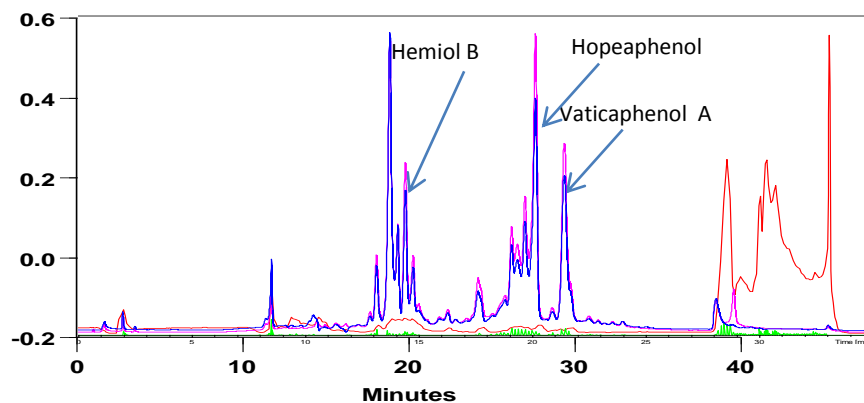


Figure 1: The identified compounds in the crude extract

Isolation and Purification of Identified Compounds

The chromatogram from HPLC analysis of crude extract shows 6 major compounds in the extract (Figure 2). Isolation of these compounds lead to 6 different fractions, however some of the fractions were impure. Fig. 3 shows chromatograms of all fractions. All fractions with impure compounds underwent further HPLC analyses for compounds purification. All, including the anticipated known compounds were isolated.

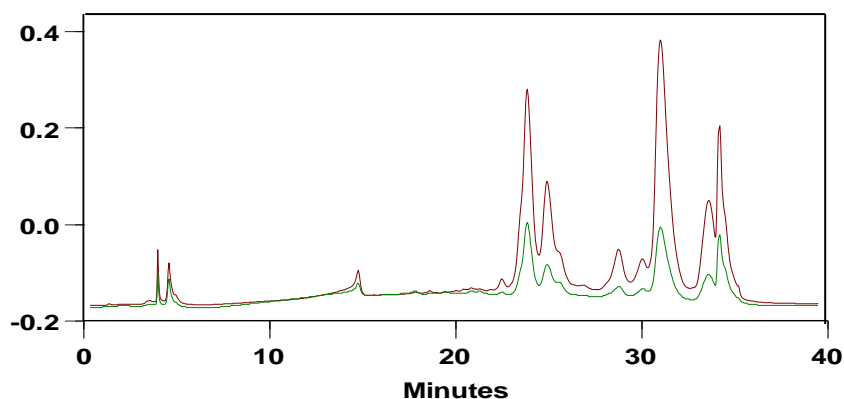


Figure 2: The chromatogram shows six well-isolated peaks

Identification of Isolated Compounds

Since all isolated compounds are known, the identification was done by comparing the $^1\text{H-NMR}$ with the reported data. Only four compounds were successfully identified due to their spectroscopic analyses and the others were insufficient for identification. The identified compounds are as follow:

Balanocarpol: It was isolated as a brown solid. Positive ion ESI-MS showed a protonated ion at m/z 471, suggested that the compound is a dimeric oligostilbene. In Figure 3a, the $^1\text{H-NMR}$ spectrum shows two sets of ortho-coupled aromatic protons in AA'BB' spin systems resonating at δ 7.48, 6.94 (2H, d, $J=8.5$ Hz, each) and 6.74, 6.41 (2H, d, $J=8.5$ Hz, each) assignable to two independent 4-hydroxyphenyl groups. Two sets of meta-coupled aromatic protons at δ 5.95, 6.08 (1H, d, $J=1.9$ Hz, each) and δ 6.25, 6.20 (1H, d, $J=1.9$ Hz, each) assignable to two disubstituted resorcinol moieties resonated. The above data fully match those published for this known dimer.⁶

Vaticanol A: It was obtained as a pale yellow amorphous powder. The compound gave an $[\text{M}+\text{H}]^+$ ion peak at m/z 681 in positive ion ESI-MS corresponding to the molecular formula $\text{C}_{42}\text{H}_{32}\text{O}_9$, which is compatible with a trimeric oligostilbene. The analysis of $^1\text{H-NMR}$ spectrum, as in Figure 3b showed the presence of six signals in the form of pseudo doublets typical of AA'BB' aromatic systems at δ 7.25, 6.81 (2H, d, $J=8.5$ Hz, each), δ 7.04, 6.57 (2H, d, $J=8.5$ Hz, each) and δ 6.52, 6.34 (2H, d, $J=8.5$ Hz, each). They were assignable to three 4-hydroxyphenyl groups. Further analysis of the $^1\text{H-NMR}$ spectrum revealed one set of signals due to a 3,5-dihydroxyphenyl group at δ 6.25 (2H, d, $J=2.0$ Hz) and 6.14 (1H, t, $J=2.0$ Hz). Signals from a set of meta-coupled aromatic protons of a 1,2,3,5-tetrasubstituted benzene ring was observed at δ 6.07 and 6.46 (1H, d, $J=2.1$, Hz, each). An aromatic proton signal of a 1,2,3,5,6-pentasubstituted benzene ring was observed at δ 6.20 (1H, s). The remaining two signals of aliphatic protons at δ 5.15 and 4.17 (1H, brs) did not show any correlation. This observation could be explained from the shape of the signals, which showed very small coupling constant, suggesting the dihedral angle to be close to 90° making the correlation becoming very weak, beyond detection by this experiment.

From all the above data, we concluded that compound is vaticanol A. This compound was first isolated from *Vatica rassak*⁷ and the above data fully matches those published for this trimer.

Vaticaphenol A: The compound was isolated as a brown amorphous powder. It showed a $[M+H]^+$ peak at m/z 907 in the LC-ESI-MS positive ion mode compatible with a molecular formula of $C_{56}H_{42}O_{12}$, suggested a tetrameric oligostilbene. In Figure 3c, the 1H -NMR spectrum shows four sets of ortho-coupled aromatic protons in AA'BB' spin systems at δ 7.22, 6.76 (2H, d, $J=8.5$ Hz, each), 7.16, 6.76 (2H, d, $J=8.5$ Hz, each), δ 7.14, 6.67 (2H, d, $J=8.5$ Hz, each) and 6.49, 6.38 (2H, d, $J=8.5$ Hz, each) resulting from the presence of four monosubstituted p-hydroxyphenyl groups. Two sets of meta-coupled aromatic protons in AX spin systems resonated at δ 6.10, 6.27 (1H, d, $J=1.7$ Hz, each) and δ 6.46, 6.17, (1H, d, $J=1.8$ Hz, each) were assignable to two disubstituted resorcinol moieties. Two signals of meta-coupled aromatic protons in AA'B spin system were observed at δ 6.08 (2H, brs), 6.27 (1H, d, $J=2.4$ Hz) suggesting the presence of an independent 2,5-dihydroxyphenyl group. Finally, an aromatic proton of a penta-substituted benzene ring resonated at δ 6.03 (1H, s). The analysis of the 1H NMR spectrum also revealed eight aliphatic methine groups. Two pairs of signals at δ 5.75, 4.42 (1H, d, $J=11.6$ Hz, each) and 5.35, 4.66 (1H, d, $J=5.1$ Hz, each) were attributed to two diaryl-dihydrobenzofuran moieties. The other two pairs of aliphatic 1H NMR signals, at δ 5.19, 3.10 (1H, d, $J=3.5$ Hz, each) and 4.08, 4.52 (1H, d, $J=10.7$ Hz, each) showed correlations as one spin system. These data are compatible with those of compound isolated from *Vatica diospyroides*.⁸

N-Acetylgalactosamine: Based on the 1H -NMR spectrum shown in Figure 3d for this compound, it shows that, it does not exhibit oligostilbene peak pattern and we concluded that it is not an oligostilbene. Through literatures search,⁹ the pattern for the spectra is likely identical with N-Acetylgalactosamine, a type of amino sugar.

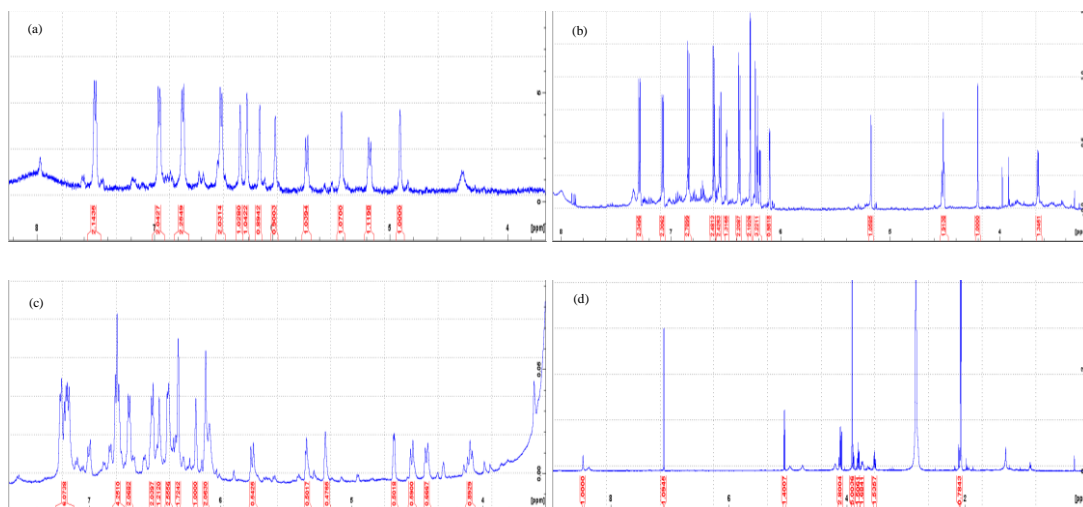


Figure 3: The 1H -NMR spectrum of (a) balanocarpol; (b) vaticanol A; (c) vaticaphenol A and (d) N-Acetylgalactosamine

CONCLUSIONS

The analysis of the isolated oligostilbenes with LC-MS ion trap system showed the competency of the system to distinguish oligostilbene directly from a crude extract. The system was recognized as being able to successfully identify a known compound solely from its fragmentation pattern, regardless of the retention time, or other data. This allows to eliminate the dependence on the chromatographic conditions and selection of column.

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