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New *E*-miyabenol isomer isolated from grapevine cane using centrifugal partition chromatography guided by mass spectrometry

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ABSTRACT

Stilbenoids have received increasing attention over the last two decades since the discovery of resveratrol in wine. With an ever-growing rhythm, a multitude of biological activities of naturally occurring stilbenes are being reported. In this work, we investigated minor stilbenoid compounds from *Vitis vinifera* stalks. The compounds were purified by means of centrifugal partition chromatography (CPC), a countercurrent-separation technique. Electrospray ionization–ion trap mass spectrometry (ESI–IT-MS) after optimization proved to be extremely efficient for the detection of these new molecules, providing both structural information for structure elucidation and a means to achieve identification even with minute amounts. Here a new stereoisomer of *E*-miyabenol C, *E-cis-cis*-miyabenol C (**2**), along with the already reported *E-trans-cis*-miyabenol C (**1**) and *E-cis-trans*-miyabenol C (**3**), was purified from a complex *Vitis vinifera* cane extract, using adapted solvent systems K and L from the 'Arizona' solvent system range, without the need for any solid support. Moreover, compounds **1**–**3** showed an inhibitory activity on α-synuclein filament formation.

1. Introduction

Stilbenes are encountered in a rather limited number of families. At present, around 30 plant families are known to produce these phenylpropanoid compounds.[1,2] Among stilbenes, *E*-resveratrol, a substance readily available commercially and therefore widely studied, has shown biological activities in protection against cardiovascular diseases and different types of cancers.[3–5] Wine and grapes are the principal dietary sources of these compounds,[6–9] and are believed to play an important role, as shown by epidemiological studies, in the prevention of cardiovascular diseases, cancer and dementia.[10] However, the low selectivity index of the small stilbenoids is of major concern. Thus, the characterization of new members of this bioactive family of compounds in *Vitis vinifera* is of extreme importance, and this is especially true for oligomers. To obtain sufficient quantities of stilbenes in order to test their biological activities in vitro and in vivo, mass spectrometry-guided purification of oligostilbenes was undertaken from *V. vinifera cv* merlot cane extracts using centrifugal partition chromatography (CPC). In this study, two quaternary Arizona solvent systems (*n*-heptane/EtOAc/MeOH/Water)[11] were successfully used to isolate three trimers of resveratrol from *V. vinifera* canes, along with several other stilbenic compounds.

The study focuses on three trimers (Fig. 1), namely *E-trans-cis*-miyabenol C (**1**), *E-cis-trans*-miyabenol C (**3**) and a new stereoisomer of miyabenol C *E-cis-cis*-miyabenol C (**2**), reported for the first time. A mixture of these three trimers was purified by CPC, thereby eliminating absorption on a solid support and recovering a greater amount of these compounds much more rapidly. These compounds were then purified using semi-preparative HPLC as a final step. Recent studies have indicated that polyphenols inhibit the formation of filaments of the protein α-synuclein (aS), which is a neuropathological feature of Parkinson's disease.[12] The inhibitory potential of compounds **1**–**3** on aS filament formation showed promising activity.

2. Results and discussion

The M*t*BE extract seemed (as calculated by UV-absorption) to contain at least 80–90% of these particular phenolic compounds

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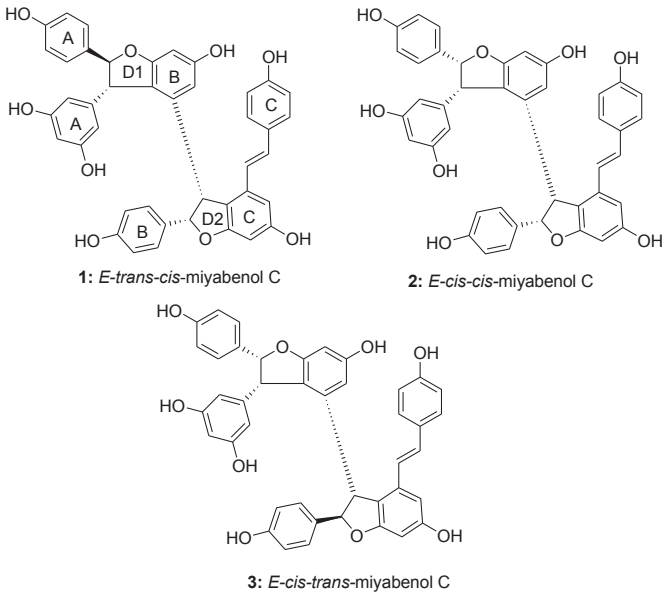

Fig. 1. Chemical structure of miyabenol isomers **1** to **3**.

(Fig. S1). Other solvents such as EtOAc, which are usually directly used after defatting the initial extract, and various other techniques allow the whole stilbene content of the plant to be isolated but are time-consuming and may lack specificity.[13,14]

2.1. Optimization of MS ionization and detection parameters

Several pure stilbenes were used as model compounds for optimizing various ionization and ion optical parameters: monomers, *E*-resveratrol, *E*-piceid, *E*-piceatannol, dimers, *E*-ε-viniferin, pallidol and a tetramer, hopeaphenol, as long as flavanoids, catechin and epicatechin were used for these experiments. *E*-ε-viniferin ionization parameters in the positive (+) mode were effective for signal enhancement of almost all stilbene oligomers. Although the negative mode provided a lower limit of detection, the fragmentation patterns were more specific in the positive mode. To simplify the operating procedure and be able to compare the different fractions, these conditions remained identical throughout the whole study. While excellent ionization and sensitivity of the analysis for oligomers were obtained in the positive mode, the monomeric hydroxystilbenes, although still visible, displayed a far higher response. This was mostly due to the mass-range window chosen to enhance the oligomer signals. These conditions gave satisfactory results for flavonoids for them to be selected for the routine analysis of all our extracts.

The following MS conditions were used for ionization, desolvation, focusing and detection: Spray Voltage +4.5 kV, Sheath Gas Flow Rate 23 L/min, Auxiliary Gas Flow Rate 3 (ratio sheath gas/auxiliary gas), Heated Capillary Temperature 220 °C, Capillary Voltage 26 V, Tube Lens Offset 45 V, and Scan Range *m*/*z* 150-2000. Helium (He) was used as the collision gas and nitrogen (N_2) as the nebulizing gas.

Data was obtained both in positive and negative ionization mode, but for most of the compounds and almost all hydroxystilbenic compounds, they were identified by their positive fragmentation pattern.

The limit of detection (LOD) was 0.1–0.3 μg/ml, which was comparable to that in other studies,[15] thus allowing the selective ionization of stilbenes and enabling detection of minor compounds. This powerful ionization and detection technique enabled acquisition of MS^8–MS^9 fragmentation spectra of all the compounds and a large amount of structural information.

2.2. CPC solvent system selection

Choosing an appropriate biphasic system is of upmost importance in CPC purification.

The 'Arizona' solvent system family is one of the most widely used together with the 'HEMWat' solvent system family. The latter was the first to be developed using *n*-hexane–EtOAc–MeOH–water in rational fixed proportions.[16] The 'Arizona' was developed by Foucault et Chevolot,[11] who replaced *n*-hexane with *n*-heptane (*n*-heptane–EtOAc–MeOH–water). 23 letter-named steps lead from polar A (0:1:0:1 v/v) to non-polar *Z* (1:0:1:0 v/v) system. The system covers a wide range of varying polarity biphasic systems and subsequent compound partition. The systems K (*n*-heptane/ethyl acetate/methanol/water 1:2:1:2 v/v) and L (*n*-heptane/ethyl acetate/methanol/water 2:3:2:3 v/v) were tested to find an appropriate solvent system to partition the Methyl *Tert*-Butyl Ether (MTBE) extract containing the majority of the hydroxystilbenic oligomers. The differences were between the two system families were minimal so 'Arizona' system K was chosen for the first partition of the MTBE extract as its stationary phase retention was better.

2.3. Purification of targeted compounds

The soluble MTBE extract was subjected to CPC separation and 22 fractions were obtained, 14 in ascending mode (aqueous-lower phase as the stationary phase) and 8 in descending mode (Fig. S2). All of these fractions presented a large number of hydroxystilbenes that were detected despite their low concentrations, thanks to our optimized ESI-MS ionization and detection parameters.

One of the ascending mode fractions of the CPC1, Fr.5 (0.81 g) mainly contained one of the major vine hydroxystilbenes, *E*-piceatannol, as well as three resveratrol trimers with quasi-molecular ions of 681 $[M+H]^+$ (Fig. S3). This fraction was then subjected to a second CPC step using the L solvent system (see chromatogram Fig. S4). This yielded 18.3 mg of a pure mixture of three trimers in tubes 126–129 (Fig. 2) at the end of the descending mode.

2.4. Structure elucidation

The fragmentation of the resveratrol trimers in the ion trap confirmed their structure, as shown in Fig. S5. The fragmentation patterns of the three resveratrol trimers were quasi-identical. The major fragments $681(MS^1)>575(MS^2)>481(MS^3)>371(MS^4)$ corresponded apparently to a first departure of a monohydroxylated phenolic ring B along with the attached carbon of D2 furan core (MS^2), the departure of the monohydroxylated phenolic ring A from D1 furan core (MS^3) and the departure of the other dihydroxylated phenolic ring A from the same furan core D1 phenolic ring (MS^4). No differences in intensity of the fragments were observed (Fig. S5). Their fragmentation patterns thus confirmed that these compounds have the same planar structure. Recent studies have demonstrated the utility of collisionally induced fragmentation for the structural determination of these compounds, compared to other techniques[17,18]

Compounds **1**–**3** were purified as yellow pale amorphous powder by semi-preparative reverse-phase HPLC. Their negative-mode ESI-HRMS spectrum exhibited a quasi-molecular $[M-H]^-$ ion at *m*/*z* 679.1973, 679.1973 and 679.1974, respectively. Considering the mass accuracy specifications of the instrument and the isotopic ratios (ca. 47%), a molecular formula of $C_{42}H_{28}O_{10}$ was determined for these three compounds. This data suggested that they could be the resveratrol trimers. Their ^1H and ^{13}C NMR data (Table 1) was unambiguously assigned by the interpretation of $2D^1H$–^1H–COSY, ^1H–^{13}C–HSQC, and ^1H–^{13}C–HMBC experiments.