CYTOTOXIC FLAVONOIDS FROM THE BARK OF *Lonchocarpus haberi* FROM MONTEVERDE, COSTA RICA

Vogler B, Cholewa L, Schmidt J, Setzer W

Department of Chemistry, University of Alabama in Huntsville
Huntsville, Alabama 35899, U.S.A.
bvogler@chemistry.uah.edu

Summary

Introduction: Higher plants serve as important sources of new drugs. About 80% of the world's population rely predominantly on plants and plant extracts for health care. There are more than 120 important prescription medicines in the United States based on plant-derived drugs (about 25% of the total). Screening for new compounds, however, poses the challenge of repeatedly finding already known compounds with previously described activity. To cope with this challenge hyphenated chromatographic methods like high-performance liquid chromatographic – mass spectrometry (HPLC-MS) have been used as a dereplication tools. It has also been successfully demonstrated to use high-performance liquid chromatographic – nuclear magnetic resonance spectroscopic (HPLC-NMR) as a dereplication tool. **Materials and Methods:** The crude acetone bark extract of *Lonchocarpus haberi* (Fabaceae, Papilionoideae) showed *in-vitro* cytotoxic activity against Hep G2, PC-3, and Hs578T human tumor cell lines. To demonstrate the feasibility of HPLC-NMR as a derepliction tool we analyzed the crude extract of *Lonchocarpus haberi* (Fabaceae, Papilionoideae) with HPLC-NMR methods. **Results:** Analysis of the HPLC-NMR spectra revealed a set of prenylated aromatic compounds. Subsequent bioassay guided isolation and characterization using 2D-NMR methods confirmed the proposed structures that indicated it to be composed largely of flavonoids. **Conclusion:** Using information rich HPLC-NMR methods can significantly reduce the time to correlate biological activity with chemical structure. Furthermore it reduces the amount of sample necessary for the study manifold.

Key Words: *Lonchocarpus haberii*, Costa Rica, cytotoxicity, dereplication, HPLC-NMR.

The isolation and characterization of new natural products generally is associated with multiple separation steps. Typical procedures require to separate multigram amounts of extracts by column chromatography. For the subsequent structural and biological characterization of pure compounds typically a few milligrams of material are required in order to perform tasks such as biological assays and structure elucidation by NMR, MS, and X-Ray methods. Generally the characterization by NMR is considered to be the least sensitive method, where nowadays a few milligram of sample is needed to measure all the required spectra. Purification, however, in general is associated with several separation and characterization steps. Large amounts of samples have to be processed and in many cases

researchers end up finding already known compounds or end up with samples with no or little activity. As a result valuable resources have been wasted.

The application of bioassay guided isolation protocols has been used to focus on biologically active compounds, while HPLC-MS applications for the characterization of mixtures of unknown compounds has been shown to be a powerful method, in order to reduce finding already known compounds (1). It has to be noted, however, that for a reliable structure determination certain sets of conditions have to be tested. Even atmospheric pressure ionization or electrospray ionization techniques (APCI or ESI) offer only limited structural information. This drawback can be improved by HPLC-MS-MS measurements However, these MS-MS spectra are not comparable to those recorded by electron impact mass spectrometry (EI) and this hampers direct use with standard natural products libraries. As a consequence specific HPLC-MS-MS libraries have to be built up, and this greatly limits this approach to small groups of compounds, that have been previously studied.

Furthermore, NMR is generally considered to be the ultimate tool for the characterization of organic compounds. One important reason seems to be that inherent connectivity information in NMR spectra, which by several "filtering" techniques, such as COSY, gHSQC, gHMBC, or NOESY, can be broken up into information packages, is important for the compound under study. NMR certainly offers the richest structural information and is applicable to almost any compound without any a-priori knowledge of that particular compound. Furthermore NMR offers the additional advantage that stereochemical differences between various compounds can be evaluated.

Since HPLC-NMR became commercially available around 1997, a large number of applications of HPLC-NMR (2), especially in natural products research, have been published. It appears that foremost European groups, like Prof. Albert, Tuebingen (3, 4, 5), Prof. Hostettmann (6, 7, 8), Prof. Bringmann (9, 10, 11), just to name the probably most active groups, have put the application of HPLC-NMR, quite often in combination with HPLC-MS, into a new light. These authors as well as ourselves (12, 13, 14, 15) have demonstrated the application of HPLC-NMR to a wide range of natural products using only very little material.

Methods

Plant Collection. *Lonchocarpus haberii* was collected from Monteverde, Costa Rica, and identified by William A. Haber, Missouri Botanical Garden. Crude extracts were obtained by exhaustive extraction using acetone as a solvent.

High Performance Liquid Chromatography Diode Array (HPLC-DAD). The crude acetone extract of *Lonchocarpus haberii* was dissolved in methanol and subjected to HPLC-DAD analysis using a Waters SunFire ^{TM} –C_{18} reversed phase column, 4.6x250 mm. Solvent composition was optimized at a flow rate of 1mL/min. Optimum separation was achieved with the following solvent gradient: 0 - 25 min: 58% acetonitrile in water; 25 – 42 min: 58% to 80% acetonitrile in water; 24 – 48 min: 80% to 100% acetonitrile; 48 – 53 min. 100 % acetonitrile.

HPLC-NMR analysis. HPLC-NMR analysis was performed on a Varian Unity Inova 500 MHz system equipped with an indirect detection flow probe, active volume 60 μL, using 50 μL injections at a concentration of 20 mg/mL of crude extract in methanol. For HPLC-NMR analysis water was replaced with 99.5% D_2O. On-Flow HPLC analysis (see figure 1) using WET solvent suppression, a scout scan to track changes in solvent composition, and 32 scans per time increment allowed the analysis of the respective HPLC-NMR spectra. Under stop-flow conditions more sophisticated WETGCOSY and WETNOESY experiments from the Varian Pulse Library were used to obtain more detailed structural information.

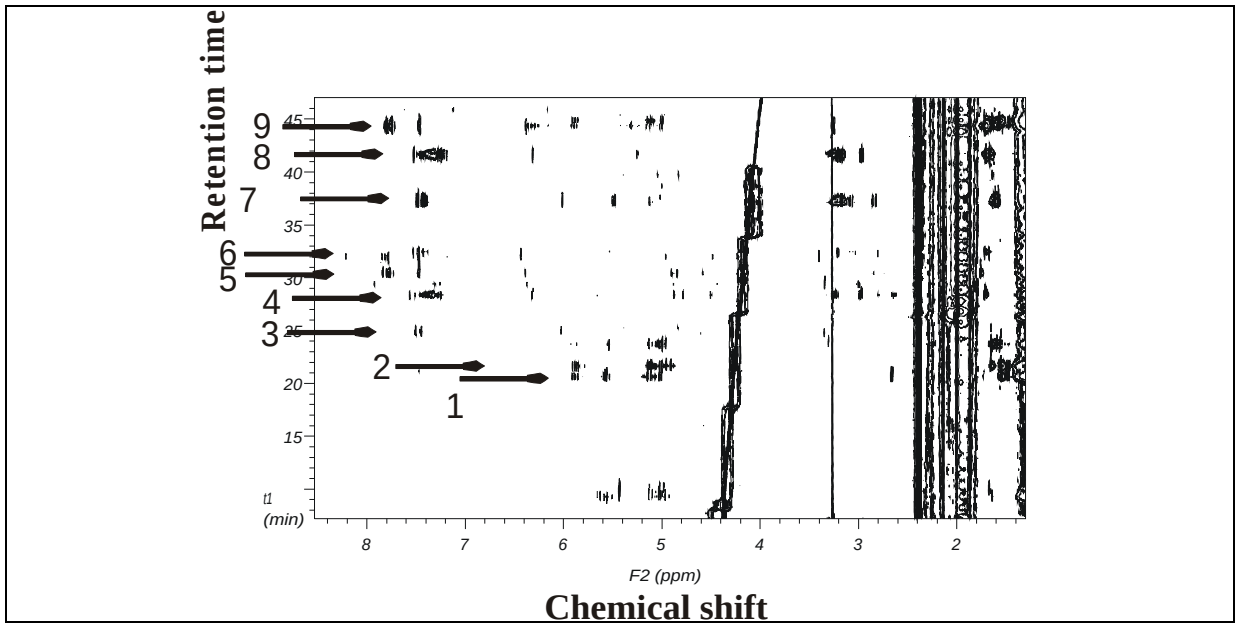

Figure 1: HPLC-NMR analysis of *Lonchocarpus haberii*. Experimental details see methods.

Isolation. Individual compounds were isolated using the same separation conditions. Fractions were collected in 1mL aliquots and evaluated with the cytotoxicity assay. Fractions that showed significant activity were further evaluated using 1D and 2D NMR methods.

NMR methods: Samples that showed significant activity were subjected to NMR analysis using deuterochloroform as a solvent. The structure determination was accomplished with a series of NMR experiments consisting of 1D ^{1}H-NMR and ^{13}C-NMR, and 2D gCOSY, gHSQC, gHMBC, NOESY experiments, all part of the Varian VNMR 6.1C software package.

Cytotoxicity Assay. Cytotoxicity of test compounds on tumor cell lines were measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulpho-phenyl)-2H-tetrazolium, inner salt] assay (15, 16, 17) for cell viability. This assay measures the ability of mitochondrial enzymes in live cells to convert the MTS to a visible formazan dye. Cells were plated in 96-well plates at a density of 1.8 x 10^4 cells per well in 100 μL.

After 48 hours incubation at 37°C, test compounds were added to quadruplicate wells and incubated with the cells for an additional 48 h. Promega Cell Titer 96® Aqueous One Solution containing MTS wais added to the cultures and the assay performed according to the manufacturer's directions. A Molecular Devices SpectraMAX Plus microplate reader was used to determine the absorbance at 490nm, and the percent of cells killed compared to the dimethylsulfoxide (DMSO) control. Doxorubicin was used as a control. The same assay can be performed with chromatographic fractions of plant extracts during the activity-guided separation and with isolated compounds to determine IC_{50} values. Crude extracts were tested at 100 μg/mL, chromatographic fractions are tested at 25 μg/mL, and pure compounds were serially diluted in order to determine IC_{50} values.

Table 1: Cytotoxicity (LC_{50}, μg/mL) of compounds from *Lonchocarpus haberii*.

	Activity		
Compound[a]	Hep G2	PC-3	Hs 578T
1-4	471(210)	> 1000	> 1000
5	322(5)	287(46)	442(101)
6	75.8(16.0)	31.3(0.1)	79.5(0.8)
7	65.3(7.3)	31.4(0.1)	79.4(1.3)
8	183(3)	37.5(1.7)	80.0(2.0)
9	97.0(16.0)	48.1(5.4)	81.5(0.5)
doxorubicin	1.32(0.23)	37.0(5.1)	10.4(1.2)

a: compound numbers refer to fractions, or traces in HPLC-NMR, see above.

Results

HPLC-NMR revealed nine chromatographically distinguishable peaks. Characteristic for the individual NMR traces was a set of methyl resonances at 1.6 ppm that accounted for either two or three methyl groups. Furthermore all traces showed a signal at 3.3 ppm (2H) that was correlating in WETCOSY analysis with a signal at 5.2 ppm. The latter signal showed also a correlation to one of the aforementioned methyl signals. Based on this analysis this group of signals was assigned to an isoprene side chain attached to an aromatic ring system. In addition COSY analysis displayed a three spin system at 5.5 ppm (1H), and a pair of doublet of doublets at 3.2ppm and 2.8 ppm. This was attributed to the proton signals of a lactone ring system. Lack of resonances for this spin system in some traces, and display of additional olefinic resonances suggested ring opening of the lactone ring. Signals at 7.2 ppm (5H) were assigned to a phenyl group, and finally a singlett (1H) at around 6.1 ppm was assigned to an aromatic proton with two neighboring phenolic hydroxyl groups. Variation in the chemical shifts of the latter proton suggested that the isoprene unit could be attached to either the 6 or 8 position of the flavonoid ring system. Traces that accounted for three methyl groups revealed additional signals at 2ppm, based on WETCOSY analysis. This strongly suggested a geranyl side chain, instead of an isoprene unit.

Bioassay guided fractionation led to the isolation of four pure compounds that showed significant activity (see table1). NMR analysis of these fractions confirmed the following structures.

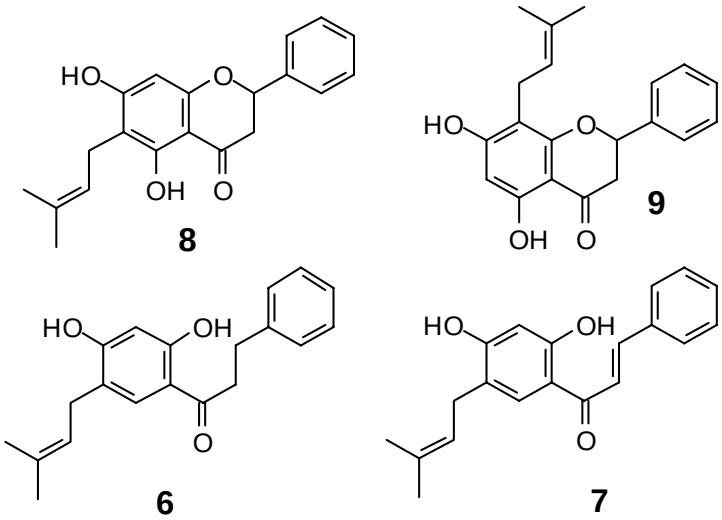

Figure 2: Structures assigned by NMR spectroscopy. Numbers refer to fraction number.

Discussion

A mixture of cytotoxic flavonoids account for the biological activity of *L. haberi* crude bark extract. It could be demonstrated that HPLC-NMR analysis quickly can reveal detailed structural data. With as little as 10 mg of plant material detailed NMR data can be collected. This allows screening of small amount of plant material and hence screening of a larger number of sample.

References

1. Kerns EH, Volk KJ, Whitney, JL Rourick, RA Lee, MS. Chemical identification of botanical components using liquid chromatography/mass spectrometry. Drug Information Journal 1998; 32:471-485.
2. Albert K (Editor). On-Line LC-NMR and Related Techniques. Wiley, 2002.
3. Krucker M, Lienau A, Putzbach K, Grynbaum MD, Schuler P, Albert K. Hyphenation of capillary HPLC to microcoil ^{1}H NMR spectroscopy for the determination of tocopherol homologues .Anal Chem 2004; 76:2623-2628.
4. Xiao HB,. Krucker M Albert K, Liang XM. Determination and identification of isoflavonoids in *Radix astragali* by matrix solid-phase dispersion extraction and high-performance liquid chromatography with photodiode array and mass spectrometric detection. J Chromatogr A 2004; 1032:117-124.
5. Glaser T, Lienau A, Zeeb D, Krucker M, Dachtler M, Albert K. Qualitative and quantitative determination of carotenoid stereoisomers in various spinach samples using MSPD prior to HPLC-UV, HPLC-APCI-MS and HPLC-NMR on-line coupling. Chromatographia 2003; 57:S-19-26.
6. Waridel P, Wolfender JL, Lachavanne JB, Hostettmann K, Identification of the polar constituents of Potamogeton species by HPLC-UV with post-column derivatization, HPLC-MSn and HPLC-NMR, and isolation of a new ent-labdane diglycoside. Phytochemistry 2004; 65:945-954.

7. Ramm M, Wolfender JL, Queiroz EF, Hostettmann K, Hamburger M. Rapid analysis of nucleotide-activated sugars by high-performance liquid chromatography coupled with diode-array detection, electrospray ionization mass spectrometry and nuclear magnetic resonance. J Chromatogr A 2004;1034:139-48.
8. Wolfender JL, Verotta L, Belvisi L, Fuzzatti N, Hostettmann K. Structural investigations of isomeric oxidised forms of hyperforin by HPLC-NMR and HPLC-MSn. Phytochemical Analysis 2003; 14:290-297.
9. Bringmann G, Lang G. Marine Molec. Biotechnol. (Ed.: W.E.G. Müller), Springer Verlag, Berlin, 2003:89-116;
10. Bringmann G, Rückert M, Messer K, Schupp O, Louis AM. Use of on-line high-performance liquid chromatography - nuclear magnetic resonance spectrometry coupling in phytochemical screening studies: rapid identification of metabolites in *Dioncophyllum thollonii*. J Chromatogr A 1999; 837:267-272.
11. Bringmann G, Guenther C, Schlauer J, Rueckert M. HPLC-NMR on-line coupling including the ROESY technique: direct characterization of Naphthylisoquinoline alkaloids in crude plant extracts. Anal. Chem. 1998; 70:2805-11.
12. Spring O, Buschmann H, Vogler B, Schilling EE, Spraul M, Hoffmann M. Sesquiterpene lactone chemistry of *Zaluzania grayana* from on-line LC-NMR measurements. Phytochemistry 1995; 39:609-612.
13. Vogler B, Klaiber I, Roos G, Walter CU, Hiller W, Sandor P, Kraus W. Combination of LC-MS and LC-NMR as a tool for the structure determination of natural products. J Nat Prod. 1998; 61:175-8.
14. Setzer WN, Vogler B, Bates RB, Schmidt JM, Dicus CW, Nakkiew P, Haber WA. HPLC-NMR/HPLC-MS analysis of the bark extract of *Stauranthus perforatus*. Phytochem Anal 2003; 14:54-9.
15. Zschocke S, Klaiber I, Bauer R, Vogler B. HPLC-coupled spectroscopic techniques (UV, MS, NMR) for the structure elucidation of phthalides in *Ligusticum chuanxiong*. Mol Divers 2005;9:33-9.
16. Setzer MC. Green gold from down under: Bioprospecting for Phyto pharmaceuticals from Paluma, North Queensland, Australia. M.S. Thesis, University of Alabama in Huntsville. 2000.
17. Setzer MC, Setzer WN, Jackes BR, Gentry GA, Moriarity DM. The medicinal value of tropical rainforest plants from Paluma, North Queensland, Australia. Pharm. Biol 2001;39:67-78.
18. Setzer, MC, Moriarity, DM, Lawton, RO, Setzer, WN, Gentry GA, Haber, WA. Phytomedicinal potential of tropical cloudforest plants from Monteverde, Costa Rica. Rev Biol Trop 2003; 51:647-674.