Screening by NMR: A New Approach for the Study of Bioactive Natural Products? The Example of *Pleurotus ostreatus* Hot Water Extract

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Direct NMR screening of natural products obtained from hot water extracts of medicinal species is accomplished through STD and tr-NOESY experiments on a crude mixture and a given protein receptor. It is shown, with use of a mushroom extract as model case, that this protocol may provide a fast and simple method, particularly useful in natural products chemistry, through which to detect the presence of ligands for a target receptor.

Introduction

Molecular recognition by specific targets is at the heart of the drug discovery process. Recently, it has been demonstrated that NMR screening is ideal for finding small ligands that bind to a receptor.[1] Since NMR, unlike bioassays, requires no prior knowledge of protein function, no target-specific set up is required for its use in pharmaceutical research. Small compounds from combinatorial libraries have very frequently been scrutinized versus given receptors.[2] Here, we alternatively show that NMR-based screening can also be performed with extracts from medicinal plants; to the best of our knowledge our work describes its application to a crude, unknown natural mixture for the first time. Moreover, the use of NMR to study a crude natural extract directly can supply significant results, as already noted by Bilia and co-workers in 2001[3] and recently highlighted by Taggi and co-workers.[4] Here we introduce the use of NMR especially to identify target compounds in a crude natural mixture that can be recognised for their binding ability to a specific protein. Although classical phytochemical analyses are carried out on organic extracts, most natural remedies prepared from medicinal plants are administered as infusions or decoctions. On-line methodology (HPLC-UV/DAD/NMR/MS)[5] represents probably the most powerful approach currently used in phytochemical analyses. Nevertheless, it seems not to be an ideal technique to study a plant water extract in depth, due to the natures of the compounds extracted with water. Thus, we have chosen, as a real test case, the crude natural mixture, labelled POw, obtained from a hot water extract of *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. – oyster mushroom – as a source of possible drugs,[6] using the lectin from *Lens culinaris* as a model target protein.[7] This approach could be extendable to other, similar ligand/protein systems relevant for pharmaceutical research; DC-SIGN and Dectin-1, for example, are two lectin-like receptors that have important roles in the modulation of human immune system responses.[8,9] In both cases, the gene sequences of their carbohydrate recognition domains (CRDs) are known.[10,11] These proteins could become relevant targets for screening by NMR spectroscopy. Further research aimed towards immunomodulatory drug discovery can be planned and performed using these proteins as targets and the traditional remedies prepared from different medicinal mushroom species, known for their immunomodulatory activity,[12] as sources of natural bioactive ligands. A second example concerns research into potential drugs against cholera toxin. The B pentamer of this protein could also be used to perform screening by NMR, since this technique has been useful in deducing the structural basis of its interaction with a variety of glycomimetics.[13] In this case it could be possible to test natural remedies prepared from medicinal plants used to treat cholera infections in indigenous traditional medical (the way to prepare a herb-based medicine is usually described in ethnobotanical reportage).

Results and Discussion

STD experiments have become a robust method to detect binding of ligands to a given receptor.[14] Figure 1 (see part A) shows the results of the application of this method to the ligand/receptor system chosen here (POw/lentil lectin), with the corresponding blanks. It is evident that one or

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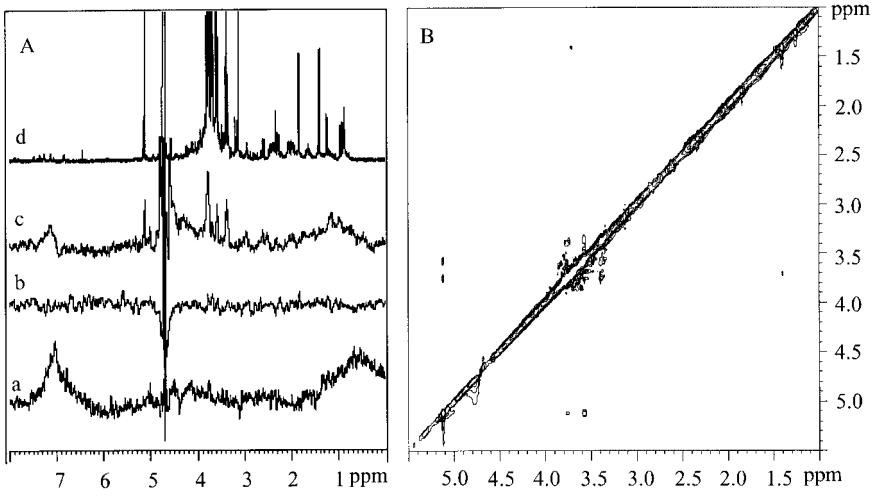

Figure 1. **A**: Comparison between NMR spectra obtained for: a) STD of lectin from *Lens culinaris* (sample **I**) without the use of spin-lock. b) STD of POw (sample **II**). c) STD of lectin with POw (sample **III**). d) ^{1}H NMR of POw. **B**: tr-NOESY of sample **III**. Cross peaks have the same sign as diagonal peaks (negative).

more compounds present in POw mixture are interacting with the lectin. Moreover, the observed chemical shifts are between 5.12 and 3.37 ppm, in the regular carbohydrate region, as expected for a lectin-interacting molecule.

The interaction of one or more ligands of POw mixture with lentil lectin was also detected by comparing the NOESY spectra (see Figure S1 in the Supporting Information) acquired for samples **II** (free POw) and **III** (POw with lectin). The proton signals previously detected in the STD analysis gave positive cross peaks (500 MHz) in the analysis of sample **II**, indicating that this bioactive compound within POw had a molecular weight of less than 1 kD. Some other sugar derivatives contained in POw showed negative signals due to their higher molecular weights (see below). In contrast, in sample **III**, the cross peaks of the interacting sugar (B in Figure 1; the intensities of the cross peaks are reduced with respect to Figure S1B in order to observe only the interacting ligand) changed to negative, as would be expected for ligand binding between some sugar-based compounds of POw with lentil lectin.[15] Analysis of these tr-NOESY cross peaks also provided information on the bioactive conformations of these compounds (see below). No chemical shift variations of the corresponding signals of the lectin-interacting molecule from the free sample **II** were observed, due to the excess of ligand with respect to the receptor used. These are the regular conditions for STD and TRNOE analysis of bioactive molecules.

The identification of the ligand was accomplished by selective STD-1D-TOCSY.[16] From the chemical shifts and coupling constant pattern, it is evident that an α-Glc moiety is present in the ligand. Further information was gained by using standard TOCSY, HSQC, HMBC and selective 1D-COSY on intact sample **II** (POw). Chemical shift values for the sugar moiety were derived from HSQC (Table S1 in Supporting Information), while the cross peak at 5.12/93.3 ppm in the HMBC (also a peak in the HSQC spectrum; see Supporting Information) indicated the presence of a trehalose unit. Apart from this moiety, an additional functional group should also be present in the ligand, due to the breaking of symmetry of the NMR spectrum at positions 5/5′ and 6/6′ of the ligand and its chemical shift differences with pure trehalose. An ester linkage is probably present, as deduced from the observed HMBC correlations (see Supporting Information). With regard to the bound conformation, further analysis of the tr-NOESY spectrum permitted deduction of the H-1/H-5′ NOE cross peak, typical of trehalose moieties in the double *exo*-anomeric conformation,[17] indicating that lentil lectin binds the major conformation of trehalose in solution. Now, the bound epitope of the bound ligand, as deduced from the STD response, may be ascribed to the face of the disaccharide defined by H-1, H-2, H-4, H-3′, and H-5′, circled in Figure 2. Obviously, knowledge of the ligand-bound conformation and ligand epitope is of key interest for drug design.[1]

Finally, and in order to verify the potential use of this NMR approach also to guide a bioassay-oriented fractionation in this real test case, the POw was extracted with methanol, providing a soluble fraction and a precipitate, labelled POw_s and POw_p respectively. ^{1}H NMR analysis (A in Figure 3) and STD experiments (Figure 4) demonstrated that the lentil ligand was retained in the soluble fraction POw_s, and not in the precipitate POw_p. Chemical shift analysis showed that POw_p fraction contained one or more saccharide molecules that could be the known polysaccharides present in the oyster mushroom.[18] The use of Diffusion Ordered SpectroscopY (DOSY) NMR experiments indicated that these molecules had higher molecular weights than those contained in the POw_s fraction, since they showed much slower diffusion coefficients[19] (B in Figure 3). Their proton signals between 4.2 and 5.4 ppm (A in Figure 3) are detected as negative cross peaks in the NOESY analysis of sample **II** (free ligands POw). Thus, NMR spectroscopic data from both 2D-DOSY and

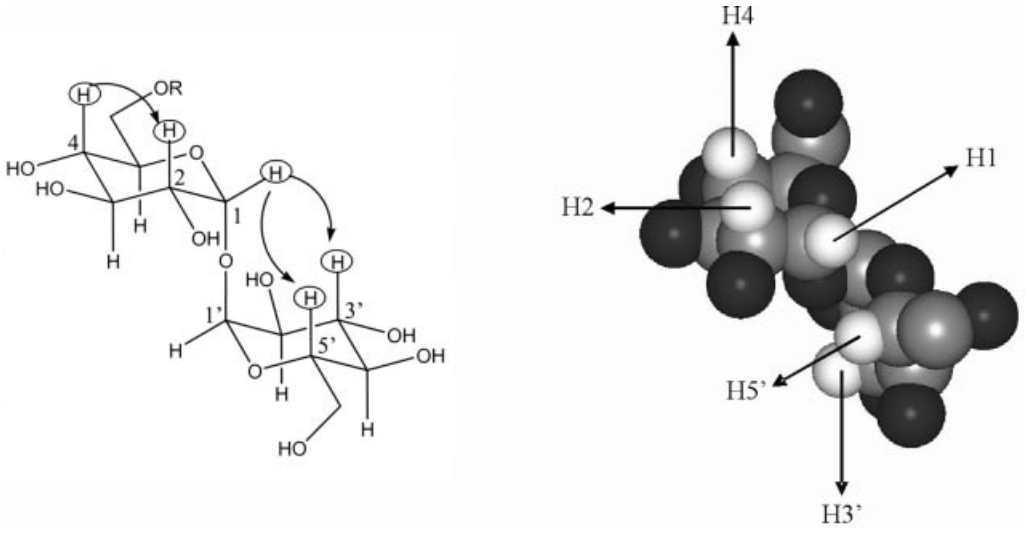

Figure 2. α-Trehalose: R = H; bioactive compound: R = unknown substituent. Circled protons are those interacting with the protein from STD experiments, also shown in the CPK model, right-hand side, in the major bioactive conformation. Arrows indicate observed tr-NOESY correlations.

NOESY are in agreement concerning the molecular weight evaluation of these noninteracting compounds within POw. In principle, further purification of the POw_s fraction could continue by separation methods until complete isolation of the ligand, with monitoring of lectin binding by simple STD experiments or just by running standard ^{1}H NMR experiments and following the properly identified key NMR signals of the free ligand.

Thus, the use of NMR for screening of crude natural product mixtures may permit useful data concerning possible ligand activity of a mixture to be obtained quickly (here in a single step), together with significant structural data on the bioactive compounds with a very small amount of material. Here, just 1 mg of the crude POw was used, an amount that could be improved through the use of modern cryotechnologies. One compound with binding ability to

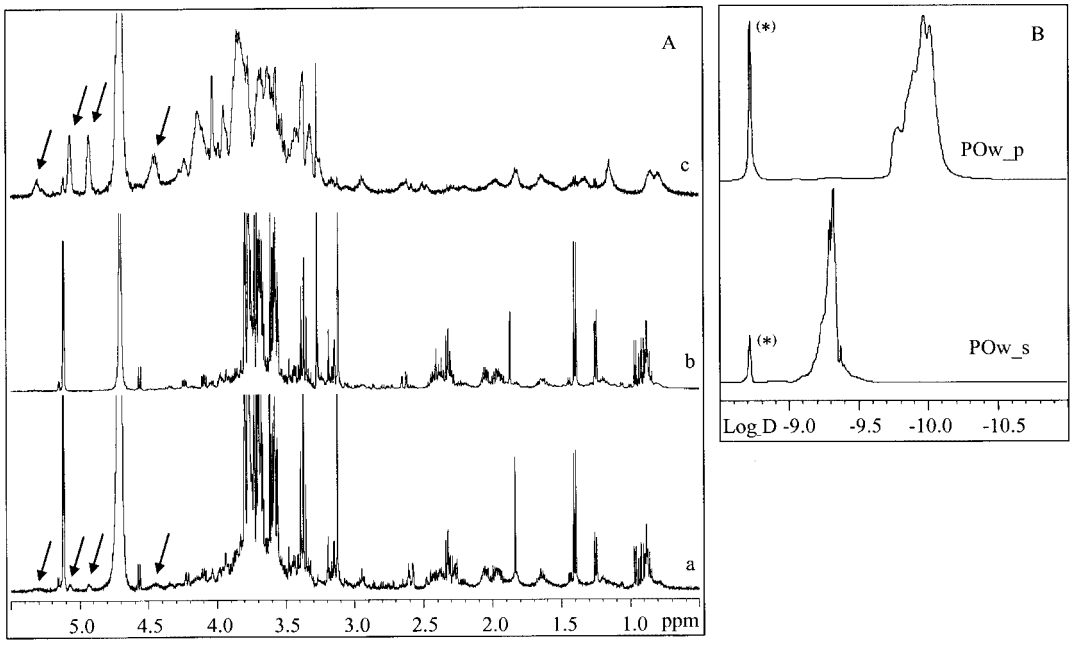

Figure 3. **A**: Comparison between the ^{1}H NMR spectra obtained for a) crude POw extract (sample **II**), b) MeOH-soluble fraction POw_s, (sample **IV**), c) POw_p fraction, precipitate in MeOH (sample **V**). Arrows highlight the low relative amount, as deduced from the intensities of the proton signals, of the compounds contained in fraction POw_p in the crude POw mixture. **B**: Comparison between the projections along the diffusion dimension of the 2D-DOSY spectra of POw_s (bottom) and POw_p (top). (*) HDO.

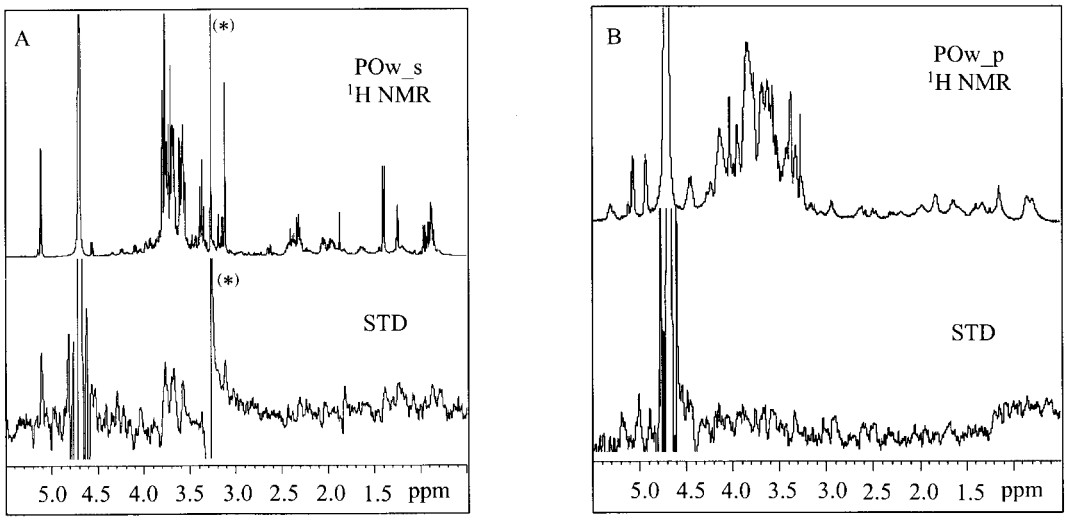

Figure 4. **A**: Comparison between STD of lectin with POw_s (sample **VI**) and ^{1}H NMR of POw_s (sample **IV**). **B**: Comparison between STD of lectin with POw_p (sample **VII**) and ^{1}H NMR of POw_p (sample **V**). (*) Methanol.

lentil lectin was easily extracted. Moreover, this method may represent a new way to guide the fractionation of a crude natural mixture, avoiding the repetition of biological testing for each purification step. In fact, a single ^{1}H NMR experiment of the fractions obtained during the separation pathways may indicate where the bioactive ligand is located.

Conclusions

The use of water as an ideal solvent for both ligands and proteins may allow ethnobotanical reportage, in which the manufacture's procedures to prepare a natural medicine from plants are described, to be better reconciled with further investigation into bioactive natural products. In principle, the approach presented here might also be applied to direct study of natural remedies from different plant species, in order to observe possible simultaneous interaction of different ligands on the same receptor (this possibility has been demonstrated with a known synthetic mixture[20]). Classical phytochemical approaches have usually avoided the study of such complex mixtures, although very often this is the usual procedure to prepare natural remedies in traditional medicine. Synergic effects have been described for many herbal remedies. Thus, this NMR approach could highlight these phenomena at a molecular level. Indeed, assignment of the proper relevance to traditional knowledge for manufacture of natural remedies might represent a new and important challenge for natural product chemists. This work may be further expanded for reevaluation of many natural medicines, administered as infusions or decoctions, so that important outputs from research on bioactive natural products may be expected.

Experimental Section

Mushroom Material: Dried fruiting bodies of *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. were purchased in a market in Madrid, Spain, in January 2004.

Extraction: The dried and powdered fruiting bodies (10 g) were extracted with 100 mL of hot water (ebb extraction) for 10 min; the extract was then lyophilised, to provide ca. 1 g of crude material, labelled as POw.

Fractionation: POw (6 mg)was extracted with MeOH to provide a soluble fraction, labelled as POw_s (yield ca. 70 %), and a precipitate, labelled POw_p.

Materials: Highly purified, salt-free, lyophilised *Lens culinaris* lectin ($MW \approx 25$ kD) was obtained from Sigma and used without any further purification. The Na_2HPO_4, NaH_2PO_4 and NaCl were of reagent quality. Before performing of the NMR experiments, the affinity of lentil lectin towards a variety of known ligands was tested.[7] It was deduced that most of the protein was intact.

Preparation of the Samples: Each NMR tube contained 0.5 mL of one of the following (**I**–**VII**) D_2O phosphate buffer solutions (75 mM, pH = 6.9), and additionally contained NaCl (75 mM).

I: *Lens culinaris* lectin (50 μM); **II**: POw (1 mg); **III**: *Lens culinaris* lectin (50 μM) with POw (1 mg); **IV**): POw_s (2 mg); **V**) POw_p (1 mg); **VI**: *Lens culinaris* lectin (50 μM) with POw_s (2 mg); **VII**: *Lens culinaris* lectin (50 μM) with POw_p (1 mg).

NMR Analyses: All the NMR analyses were performed with a Bruker Avance 500-MHz spectrometer at 298 K, equipped with a triple resonance ^{1}H, ^{13}C, ^{15}N probe. Chemical shift are in ppm with respect to the 0 ppm point of the manufacturer indirect referencing method. The saturation transfer difference (STD) experiments were recorded (number of scans 4096) by use of essentially the sequence proposed by Meyer and co-workers.[14] A cascade of soft Gaussian-shaped pulses of 50 ms (with a power level of 50 Hz) was used for the 2.5 s saturation time. On-resonance irradiation was carried out at 0 ppm, while the off resonance was set at $\delta = +50$ ppm. A short spin-lock period (15 ms) was used prior to the acquisition in order to eliminate the background protein signals, except for the pure lectin spectrum, which was acquired without the spin-lock. tr-NOESY experiments on samples **II** and **III** were performed with mixing times of 250 ms; number of scans 64. 2D-TOCSY (mixing time 70 ms) and 2D-gradient enhanced COSY were performed by standard sequences, with 256 increments of 16 scans each, separated by a 1.5 s relaxation delay, and spectral widths of 12 ppm, with two real data points in *f*2. HSQC (16 scans) and HMBC (32

scans, *J*-evolution time 60 ms) experiments on sample **II** were also performed by standard sequences. A relaxation delay of 1.5 s was used, with 256 increments, and spectral widths of 12 ppm in *f*2 and 120 ppm (HSQC) or 180 ppm (HMBC) in *f*1. Two real data points in *f*2 were acquired. Squared cosine bells were used for processing. For the 2D-DOSY experiments, the standard Bruker protocol was used for processing; with the ledbpg2s pulse sequence, and a linear gradient of 32 steps between 2 % to 95 %, and a diffusion time (big delta) of 0.2 s.

Supporting Information (see also footnote on the first page of this article): Comparisons between the tr-NOESY spectra of free POw and the mixture of POw with lentil lectin are shown in Figure S1. Only negative peaks are displayed. The exhaustive NMR analyses that allowed the partial identification of the trehalose derivative are shown in Figures S2 and S3 (1D and 2D NMR experiments, respectively). All the NMR spectroscopic data acquired are gathered in Table S1.

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