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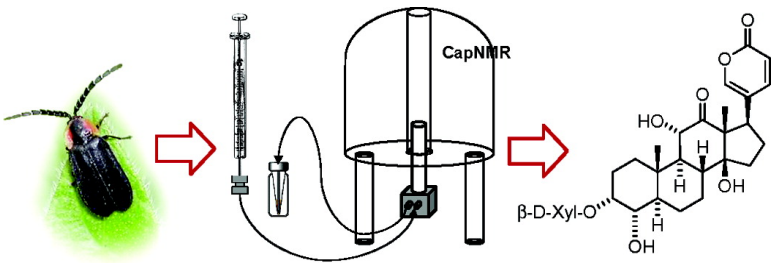

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Exploring Uncharted Terrain in Nature's Structure Space Using Capillary NMR Spectroscopy: 13 Steroids from 50 Fireflies

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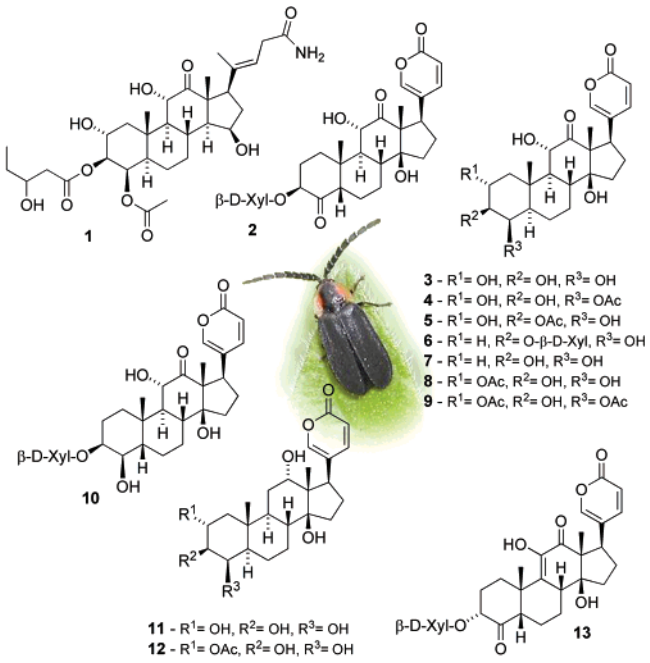
We recently described a new approach for characterizing natural product extracts based on direct NMR spectroscopic analyses of largely unpurified materials.[1] We now report progress toward lowering the threshold of material required for structural analysis of small molecule mixtures through implementation of recently developed capillary NMR probe technology[2] and demonstrate its efficiency for the identification of a large number of novel steroids from a severely mass-limited biological sample, a small collection of specimens of the firefly *Lucidota atra*.

Many natural products with profound biological activity are produced in only minuscule amounts, reflecting their natural potency. Simply because of their scarcity, many organisms with potentially interesting secondary metabolites are effectively placed off limits for natural products exploration, as insufficient NMR spectroscopic sensitivity and spectral quality severely constrain the structural analysis of mass-limited samples. Enhancing the mass-sensitivity of NMR spectroscopy while maintaining high spectral resolution would thus expand the accessible fraction of Nature's structure space significantly, thereby helping to unlock new sources of chemical diversity.

Arthropods constitute one particularly rich source of biologically active natural products, which remains largely unexplored, because collecting large numbers of specimens is often not feasible. Certain fireflies, for example, have been shown to be defended from predation by the presence of steroidal pyrones (lucibufagins) in their blood.[3] These firefly species are nocturnal, using the familiar bioluminescent signals in courtship. There are, however, less common firefly species that are active during the daylight hours and do not use light signals for mating. Such diurnal species, for example, *Lucidota atra*, may face a different array of potential predators, an ecological difference that might be reflected in their defensive chemistry.

Native to the entire eastern half of the USA, *L. atra* is relatively rare, and large numbers of individuals cannot be collected. Preliminary ^{1}H NMR spectroscopic analysis carried out on a crude sample of hemolymph collected from five *L. atra* specimens quickly revealed the presence of steroidal pyrones, as evidenced by proton resonances corresponding to the pyrone spin-system as well as by those characteristic of the angular methyl groups of a steroidal skeleton. From these initial NMR spectroscopic analyses, it appeared that the fireflies contained a large number, possibly 10 or more, of novel steroids. Although crude mixtures of complex composition can often be characterized in considerable detail without any fractionation or purification, extreme crowding and overlap of the signals of the multiple similar steroids did not allow complete characterization in this case, necessitating partial fractionation via

Chart 1. Steroids Identified from *L. Atra* Using Capillary NMR



HPLC. From the combined whole-body extracts of all 50 available specimens, 11 HPLC fractions were obtained, each containing one to three steroidal pyrones and related derivatives. The amounts (40−150 nmol) present in most of these fractions fell well below what is needed for complete structural analysis by NMR using an inverse detection probe with low-volume tubes (Shigemi) at commonly available field strengths. Cryo probes can provide significant sensitivity gains; however, the comparatively poor line shapes achieved with cryo probes complicate acquisition of the high-resolution dqf-COSY spectra needed for the analysis of mixtures and, furthermore, result in sensitivity losses for spectra, such as HMBC, that feature a long delay as part of their pulse sequences. This led us to investigate the applicability of recently developed capillary NMR probes, which had been used previously for high-throughput screening of natural products libraries.[4]The CapNMR probe[2] features a very small 5 μL flow cell with an active volume of only about 3 μL (Figure 1). The flow cell is connected to two 2 m long fused-silica capillaries serving as inlet and outlet. For loading of the flow cell, the sample, dissolved in 5 μL of deuterated solvent, is injected into the capillary via syringe, followed by injection of another 9 μL of deuterated solvent, which serves to push the sample through the entire path of the inlet capillary into the flow cell.

As part of our initial evaluation of the CapNMR system, we determined the probe to offer a 4- to 5-fold higher signal-to-noise

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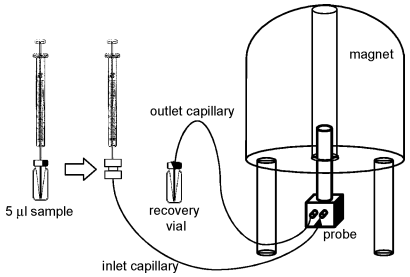

Figure 1. Sample injection and CapNMR probe.

(S/N) ratio for HMBC and HMQC spectra of a 17.2 μg sucrose sample, compared to using a standard 5 mm inverse-detection probe with Shigemi tube.[5] It should be noted that while mass sensitivity is significantly higher for the CapNMR probe, concentration sensitivity is actually only a fraction of that of 5 mm probes; by reducing sample volume from roughly 200 μL (for a Shigemi tube) to 5 μL (in the CapNMR probe), one increases concentration by a factor of about 40, while sensitivity increases only by a factor of 4−5. Nonetheless, for natural product analysis, which is almost inevitably mass-limited, the CapNMR probe seemed to be extremely promising.

The 11 HPLC fractions obtained from *L. atra* represented the first natural product samples of unknown composition for which we employed the CapNMR probe. To harness the better mass sensitivity of the CapNMR probe effectively, it was necessary to dissolve each sample in a volume as close to 5 μL as possible, taking special care to minimize losses during concentration and injection.[6]

For each *L. atra* HPLC fraction, a comprehensive set of 2-D spectra (dqf-COSY, NOESY, nongradient HMBC, and HMQC) was collected using the capillary probe. The resulting spectra allowed for complete assignment of all of the major components present in each of the fractions, leading to the identification of 13 new steroids (**1**−**13**). For most samples, all four spectra were collected within a 16−24 h period. Of particular importance for the purpose of characterizing mixtures of several structurally similar compounds was the excellent line shape and the very low level of artifacts in the spectra, which compared favorably with the much higher artifact levels commonly experienced when using cryo probe systems. For example, structures **1**, **8**, and **13** coeluted during purification and were analyzed as a three-component mixture without further purification. The low level of artifacts is likely a consequence of the small sample volume and the much higher concentration compared to 5 mm sample tubes, which keeps solvent peaks relatively small. Directly comparing CapNMR spectra of the *L. atra* fraction containing **9** with spectra obtained for the same sample using a 5 mm probe with a Shigemi tube, we determined a roughly 3-fold S/N gain (Figure 2). To complete the structural assignments, all samples were also analyzed by MS, which served to establish molecular weights and confirm the degree of heteronuclear substitution inferred from the NMR spectroscopic analyses.

All but one of the 13 compounds characterized represent steroidal pyrones analogous to those reported from other species of firefly, but with greater variation in the degree of oxidation of the steroid skeleton. Interestingly, 10 of these compounds possess a *trans*-fused A−B ring system, which has not been previously observed for steroidal pyrones isolated from insect sources. Of particular interest is structure **1**, which instead of a pyrone substituent, contains a pentenoic acid amide moiety corresponding to a ring-opened and reduced pyrone. Whether this structure represents a biosynthetic precursor or downstream metabolite of the steroidal pyrones is not known.

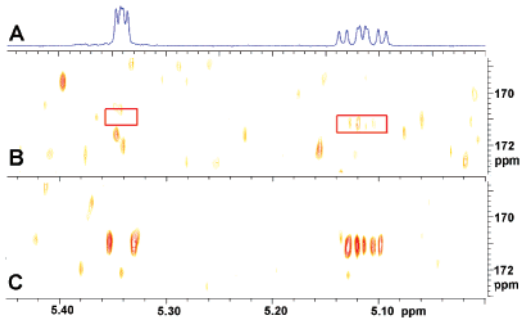

Figure 2. Sections of HMBC spectra for **9** showing HMBC correlations, indicating the location of the two acetyl groups (600 MHz). (A) Corresponding part of the ^{1}H NMR spectrum; (B) HMBC obtained using a 5 mm HCN inverse-detection probe with Shigemi tube, cross-peaks are not discernible; (C) HMBC obtained using the CapNMR probe, clearly showing the expected cross-peaks. HMBC spectra were acquired under identical conditions (see Supporting Information for details).

Considering that increasing magnetic field strength from 600 to 900 MHz results in an improvement of S/N of less than a factor of 2, the 3-fold S/N gain provided by the comparatively simple and inexpensive CapNMR system seems impressive. For example, using the CapNMR probe, HMBC spectra of amounts of the order of 40 nmol were obtained via routine overnight experiments on a 600 MHz spectrometer. As we have shown for *L. atra*, this sensitivity gain can facilitate the discovery of new, interesting chemistry, as most of the 13 steroids identified could not have been characterized in a reasonable amount of time without the CapNMR probe. It should be noted that our analysis did not involve exhaustive purification of the crude sample. As discussed previously, purification of severely mass-limited natural product extracts should aim at reducing the sample's complexity only up to a point where structure determination using NMR spectroscopy becomes possible. We expect that synergism resulting from the combination of this approach with use of the CapNMR probe will greatly extend the range of accessible natural products research.

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Supporting Information Available: Experimental conditions, example CapNMR spectra, NMR and MS data for compounds **1**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) For a comparison of CapNMR and 5 mm HCN probe with Shigemi tube using a 17.2 μg sucrose sample, see Supporting Information.

(6) For a detailed description of sample preparation for CapNMR analysis, see Supporting Information.

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