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Application of liquid chromatography–nuclear magnetic resonance spectroscopy to the identification of natural products

Steven C. Bobzin[a,*], Shengtian Yang[b], Thomas P. Kasten[a]

[a]*Searle Discovery Research, Monsanto Company, St. Louis, MO 63198, USA*
[b]*Monsanto Analytical Sciences Center, Monsanto Company, St. Louis, MO 63198, USA*

Abstract

LC–NMR combines the separation power of high-performance liquid chromatography (HPLC) with the superior structural information content of nuclear magnetic resonance (NMR). These two techniques traditionally have been the primary tools used by natural products chemists to isolate and determine the structures of molecules of interest. Recent advances in NMR technology have allowed the practical application of LC–NMR, thus providing natural products chemists with a hyphenated technique which combines the two most important tools in their field. A brief review of the literature describing how LC–NMR has been applied to natural products research is followed by a specific example illustrating how this technique was used to identify the marine alkaloid aaptamine (**1**). Aaptamine was identified as the active component in the crude dichloromethane extract of the sponge *Aaptos* sp. which had been determined to possess inhibitory activity against the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) by a high throughput screening (HTS) effort. Isolated aaptamine (**1**) exhibited an $IC_{50}=120$ μM against this enzyme. The experience gained from the identification of aaptamine was used to define a strategy for the use of LC–NMR in a natural products HTS program.

Keywords: Natural products; LC-NMR; HPLC-NMR; Dereplication; Aaptamine; GFAT; High throughput screening; HTS

1. Introduction

For the past 20 years high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR) have been the primary tools used by natural products chemists for the isolation and identification of compounds, respectively. The traditional approach has been to apply various chromatographic techniques, including HPLC, to isolate a few milligrams of a pure natural product followed by mass spectrometry (MS) and NMR experiments in a 5 mm or 3 mm tube to determine the structure of a molecule of interest. This strategy is time consuming and frequently results in the realization that the desired compound had been previously reported in the literature after a great deal of time and resources have been invested.

The ability to rapidly identify known or undesirable compounds in natural products extracts is a critical step in an efficiently run natural products discovery program. This process, commonly called dereplication, is important to prevent the unnecessary use of resources on the isolation of known or undesirable compounds from extracts identified by

*Corresponding author. Monsanto Company, Nutrition and Consumer Sector, 800 North Lindbergh Boulevard, Mail Zone T4L, St. Louis, MO 63167, USA. Tel.: +1-314-6943-541; fax: +1-314-6944-977.

E-mail address: steven.c.bobzin@monsanto.com (S.C. Bobzin).

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the screening process. This prevents wasted efforts on samples with no potential for development and allows resources to be focused on the most promising leads. The recent application of high throughput screening (HTS) technologies to assay natural products extracts for biological activity has intensified the need for efficient dereplication strategies. The number of samples identified and the timelines dictated by modern HTS programs require a rapid and effective prioritization of extracts.

Dereplication strategies employ a combination of separation sciences, spectroscopic methods [1], and database searching [2]. HPLC has been by far the most useful tool for the separation of complex mixtures of small molecules. Reversed-phase HPLC on octadecylsilane (ODS or C_{18}) has come to be recognized as the most broadly applicable of bonded phases for this purpose. When interfaced with diode array detection (DAD), HPLC allows an analyst to identify known compounds by comparison of their HPLC retention time and UV spectra. Unfortunately, this requires the compilation of an internal database because these parameters are not present as searchable fields in most commercially available databases. More recently, the advent of electrospray mass spectrometry (ES-MS) has provided a MS interface which is applicable to the analysis of a wide range of molecules and is compatible with liquid chromatography. In the past five years, LC–MS has become a widely used tool for the dereplication of natural products [3–5]. LC–MS has become the dereplication tool of choice because the nominal molecular mass of a compound can be used as a search query in nearly all databases. Unfortunately, database searching using only the molecular mass frequently produces large answer sets and rarely results in a definitive identification. LC–MS is often combined in series with DAD thereby providing UV data to narrow down the answer set. This data, along with the taxonomic information of the source organism, if available, often provides adequate information to narrow down the answer set to a short list, or sometimes a single answer. But, in many cases, more information is required for a confident identification.

Recently, advances in NMR have allowed HPLC to be practically interfaced directly with NMR. These advances include the use of higher field magnets (500 MHz and greater) and digital signal processing which have helped address the lack of sensitivity of this technique. In addition, new probe designs which allow the use of gradient pulse sequences now provide the efficient and specific suppression of the NMR signals due to the HPLC solvents. NMR spectral data provide a great deal of structural information about a compound of interest. The NMR signal for each proton in a molecule provides structural information about the environment and the coupling partners of that proton. Therefore, NMR easily can be capable of discerning structural differences between compounds of the same molecular mass (isobars), or even the same molecular formula (isomers). This includes the capability to discern between constitutional isomers and even geometric isomers.

With the recent technological advances, the scope of applications of LC–NMR has increased as evidenced by the many articles in this special issue on hyphenated techniques. While applications to the field of drug metabolism have become quite common ([6]; see other articles in this special issue), reports of the use of LC–NMR to identify natural products have been relatively rare. The first reports of the application of LC–NMR to natural products involved the characterization of isomeric mixtures produced by exposing a pure natural product to light or heat. LC–NMR was used to determine the structure of the photoisomerization product of the bioinsecticide azadirachtin [7] and to characterize the geometric isomers of vitamin A acetate produced upon exposure to heat [8]. The azadirachtin study employed stopped-flow LC–NMR measurements, while the vitamin A acetate study used on-flow measurements to detect the resonances of olefinic protons in order to determine *cis* or *trans* configurations of double bonds.

In the past few years, LC–NMR studies of natural products have progressed to include the characterization of individual components in crude, or partially purified, extracts. These applications demonstrate the full power of this hyphenated technique by eliminating the need to isolate individual components from a crude extract for subsequent NMR experiments. The first report of this type of application was the characterization of the sesquiterpene lactones in *Zaluzania grayana* by on-flow and stopped-flow LC–NMR experiments [9]. Since this report, LC–

NMR has been used to characterize a wide range of plant natural products, a testimony to the broad applicability of this technique. LC–NMR has been used to identify prenylated flavanones from *Monotes engleri* [10], monoterpene dimers from *Lisianthius seemannii* [11], and napthoquinones from *Cordia linnaei* [12]. In addition, napthylisoquinoline [13,14] and pyrrolizidine [15] alkaloids, sesquiterpene lactones [16,17], phenylphenalenones [18], taxanes [19], lignans [20], glycosides [21], and other compounds [22–27] have been identified by data obtained from LC–NMR experiments. Most of these reports describe the identification of known members of these structure classes by LC–NMR, but in a few cases LC–NMR has been used to determine structures of new analogues of known compounds [9–11]. In these examples, stopped-flow measurements employing two-dimensional (2D) NMR experiments such as COSY (corellation spectroscopy) [17], TOCSY (total correlation spectroscopy), GHSQC (gradient heteronuclear single quantum correlation), GHMBC (gradient heteronuclear multiple bond correlation) [10], and NOESY (2D nuclear *Overhauser* effect spectroscopy) [17] have been applied to determine structures of novel compounds. Much of this work has been summarized in recent reviews [28–30].

The application of hyphenated techniques to the study of natural products has recently progressed beyond the use of LC–MS or LC–NMR individually. Direct coupling of LC–NMR–MS has been demonstrated in the characterization of constituents of *Hypericum perforatum* [31]. LC–NMR–MS has also been used to identify ecdysteroids from *Silene otites* [32] which were not detected by LC–NMR alone [33]. It is surprising to note that nearly all of the reports of the application of LC–NMR to the field of natural products has been to define structures of plant-derived metabolites, while very little use has been made by researchers investigating microbial [34] or marine [35] natural products.

In this report we describe the identification of the marine alkaloid aaptamine (**1**) in the crude dichloromethane extract of the marine sponge *Aaptos* sp. using stopped-flow LC–NMR experiments. The extract of *Aaptos* sp. had been determined to possess inhibitory activity against the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) by a HTS program searching for inhibitors in natural products extracts. GFAT is an attractive molecular target for the discovery of drugs which may be used for the treatment of type II diabetes.

Type II (adult onset) diabetes [also called non-insulin dependent diabetes melitus (NIDDM)] is characterized by insulin resistance of glucose transport in the skeletal muscle and adipose tissue caused by defects in both glucose metabolism and insulin secretion [36]. The molecular basis by which insulin resistance is produced is a subject of great interest. One pathway which may contribute to the development of insulin resistance is the hexosamine biosynthetic pathway. Marshall et al., using a model system of primary rat adipocytes, proposed that the hexosamine pathway contributes to the development of insulin resistant glucose transport [37]. Subsequent studies suggested that the pathway may contribute to the development of insulin resistance in vivo [38–41]. Additional work has expanded the potential role of the hexosamine pathway and has suggested that it may function as a ''glucose sensor'' for aspects of glucose-dependent metabolic regulation of not only glucose transport but also glycogen synthesis, glycolysis, and growth factor expression [42]. The first and rate-limiting enzyme of the hexosamine pathway is GFAT, which catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate [43–45]. Therefore, GFAT was determined to be an attractive molecular target for a HTS effort to discover molecules which may be developed into drugs for the treatment of type II diabetes.

2. Experimental

2.1. Collection and extraction

The marine sponge *Aaptos* sp. was collected by SCUBA at a depth of 40 ft. offshore of Manado, Northern Sulawesi, Indonesia (1 ft.=30.4801 cm). The sponge tissue was frozen for storage and then lyophilized. A 1-g portion of the lyophilized tissue was powdered in a Kleco pulverizer and the powder extracted in dichloromethane (50 ml) overnight. The suspension was filtered and the solvent removed to yield an oil which was resuspended in dimethyl

sulfoxide (DMSO) at 30 mg/ml for screening. This solution was used for all subsequent experiments.

2.2. High throughput screening GFAT assay

Activity of GFAT (L-glutamine:D-fructose-6-phosphate amidotransferase; EC 2.6.1.16) was measured using a novel ion-exchange assay format. Specific details of this assay will be published elsewhere. The HTS assay was formatted in 96-well microtiter plates using a radiometric readout of GFAT activity. Six-point dilution curves of active samples were made starting with 1 μl of extract into a final assay volume of 50 μl. Appropriate controls were performed in each experiment.

2.3. Liquid chromatography–mass spectrometry

LC–MS experiments were performed using a Hewlett-Packard 1100 HPLC system interfaced with a Perkin-Elmer Sciex AP100 mass spectrometer. The mass spectrometer was operated in the positive ion mode using an ionspray interface. The potentials applied were 5.5 kV to the needle assembly, 25 V to the orifice, and 275 V to the ring electrode. These ''mild'' conditions provide minimal fragmentation and allow maximum sensitivity for the observation of molecular ions. The chromatography was performed on an Alltech Alltima C_{18} (5 μm) HPLC column (150×4.6 mm) using a solvent gradient from 10–100% acetonitrile (0.01% trifluoroacetic acid) in water (0.01% trifluoroacetic acid) in 25 min at a flow-rate of 1 ml/min. A 20-μl injection (600 μg) of the crude extract was made and the flow was split (9:1) after the UV detector with 10% going to the mass spectrometer and 90% collected in a deep dish microtiter plate (1 fraction/min). The fractions were dried on a Savant SpeedVac and then assayed for enzyme activity. Fraction 12 possessed the majority of the activity in the HPLC-bioassay profile. This activity corresponded with a peak at 10.3 min in the MS chromatogram due to the delay between detectors and the fraction collector.

2.4. Liquid chromatography–nuclear magnetic resonance spectroscopy

LC–NMR data were acquired using a Varian Unity-Inova 500 MHz spectrometer equipped with a $^{1}H\{^{13}C\}$ pulsed field gradient (PFG) LC–NMR flow-probe with a 60 μl flow-cell (active volume). ^{1}H-NMR spectra were obtained in stopped-flow mode as described in the Results section. Varian WET solvent suppression [46] and related sequences were used to suppress the acetonitrile, its ^{13}C satellites, and the residual water peaks. The WET technique used a series of variable tip-angle solvent-selective radio frequency (RF) pulses, where each selective RF pulse is followed by a dephasing field gradient pulse. Free induction decays were collected with 16K data points, a spectral width of 8000 Hz, a 3-μs 90° pulse, a 2 s acquisition time, and a 1 s pulse delay. A total of 256 transients (about 15 min acquisition time) were acquired to obtain the data in Fig. 3. Prior to Fourier transformation, an exponential apodization function was applied to the free induction decay corresponding to a line broadening of 0.25 Hz. The HPLC system consisted of a Varian 9012 solvent delivery system and Varian 9050 variable-wavelength UV–Vis detector. The outlet of the UV detector was connected via a sampling unit (Valco valve) to the LC–NMR flow probe. The HPLC method used an Alltech Alltima C_{18} (5 μm) HPLC column (150×4.6 mm) using a solvent gradient from 10–100% acetonitrile (0.01% trifluoroacetic acid) in D_2O (0.01% trifluoroacetic acid) in 25 min at a flow-rate of 1 ml/min. A 100 μl injection (3 mg) of the crude sponge extract was made for the LC–NMR stopped-flow experiment.

3. Results

A HTS assay was designed to identify inhibitors of GFAT which could be developed into drug candidates for the treatment of type II diabetes. High throughput screening of Monsanto's collection of natural products extracts identified 283 samples which exhibited inhibitory activity against this enzyme. A combination of biological and chemical prioritization schemes was used to select extracts for further characterization.

The crude dichloromethane extract of the sponge *Aaptos* sp. collected in Manado, Indonesia was identified by the HTS effort for GFAT (IC_{50}=18 μg/ml). In order to identify the active component in this crude extract, it was analyzed by LC–MS and LC–DAD methods. Fractions of the HPLC eluent

were collected during the LC–MS experiment and assayed for GFAT activity to determine which peak in the chromatogram possessed the observed activity. This effort allowed the peak at 10.3 min to be identified as the single, active component in the crude extract (Fig. 1). The mass spectral data for this peak indicated a molecular mass of 228 u and the DAD data indicated UV maxima at 237, 255, 309, and 375 nm for this compound. These parameters were used to search the marine natural products database, MarinLit [47]. Four compounds fit this data set (Fig. 2); aaptamine (**1**) [48,49], isoaaptamine (**2**) [50], aaptosine (**3**) [51], and the paragracine relative (**4**) [52]. It was clear that the data at hand could not confidently discern between these four compounds and that more information would be required to make a definitive identification of the active component in the sponge extract.

In order to obtain more structural information about the active component in the sponge extract, a stopped-flow LC–NMR experiment was conducted on the crude extract using the same HPLC column and conditions as used for the LC–MS and LC–DAD experiments. The only difference in the chromatographic conditions was the use of D_2O as the aqueous component of the HPLC solvent system. The eluent from the HPLC column was directed through a UV detector and then through the LC–NMR flow probe. The volume between the two detectors was calibrated and the solvent flow stopped after the appropriate delay following the observation of the UV peak previously observed at 10.3 min in

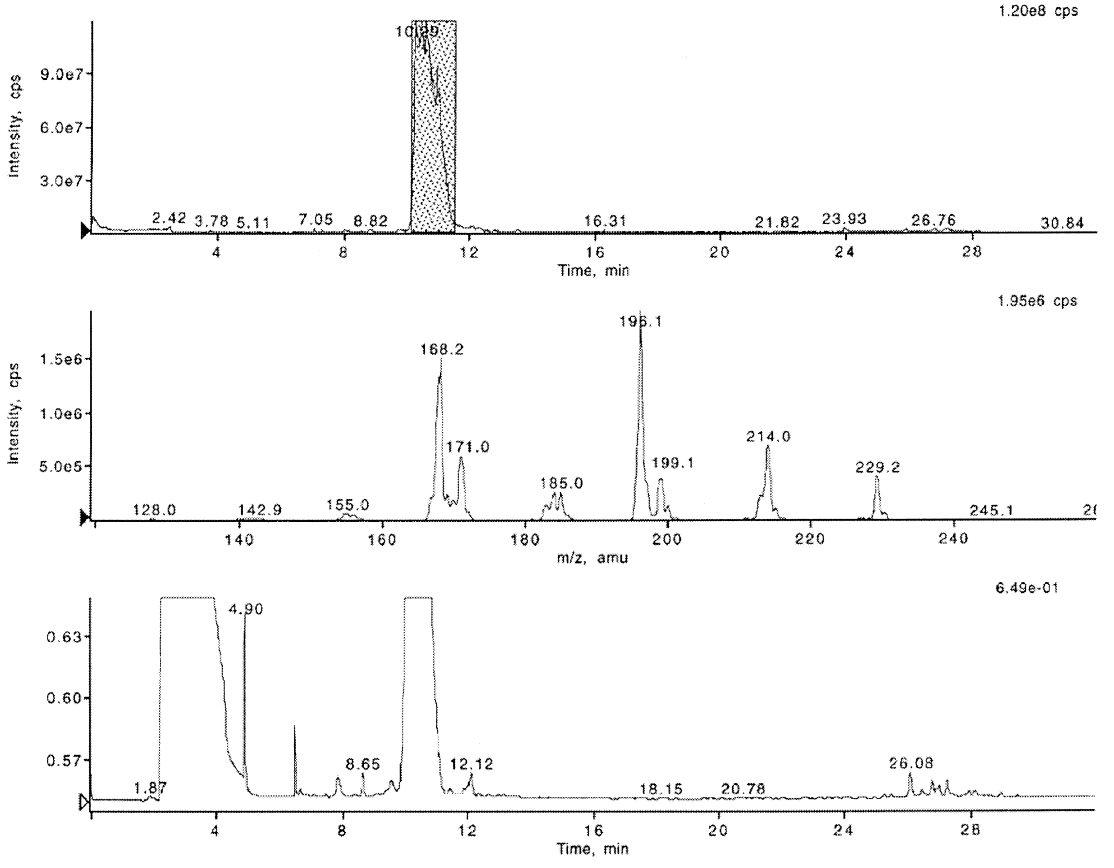

Fig. 1. LC–MS data from the separation of the crude extract of *Aaptos* sp. by reversed-phase C_{18} HPLC (see text for details). Top panel: positive ion total ion chromatogram (TIC). Middle panel: averaged, background subtracted, and smoothed mass spectrum of the peak at 10.3 min [$(M+H)^+$ at 229 u]. Bottom panel: LC–DAD chromatogram (UV at 205 nm).

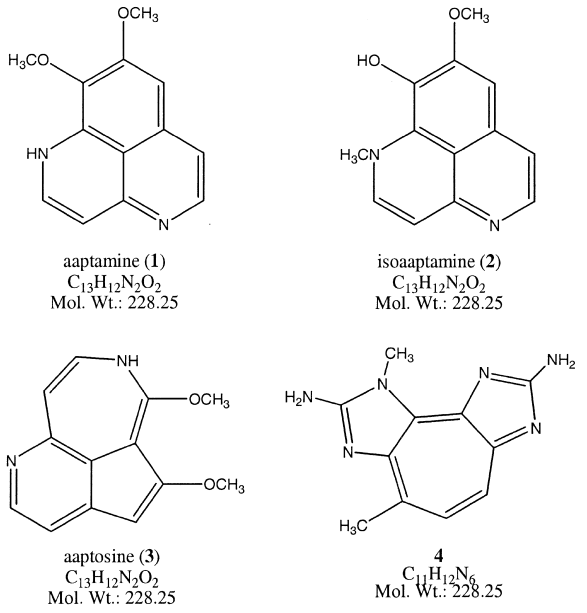

Fig. 2. Possible structures for the GFAT inhibitory component of the crude extract of *Aaptos* sp. determined by LC–MS data and database searching.

the LC–MS experiment. This placed the peak of interest in the NMR detection cell of the LC–NMR flow probe. The ^{1}H-NMR spectrum of this peak was acquired using a WET solvent suppression routine [46] to eliminate the signals due to the acetonitrile, its ^{13}C satellites, and water in the HPLC eluent. The spectrum obtained (Fig. 3) revealed four coupled NMR signals and one singlet in the aromatic region (6–8 ppm) and two large singlets at δ 3.83 and 3.97 ppm. This information could be used to eliminate the paragracine relative (**4**) from consideration because only two aromatic protons would have been observed for this molecule and the NH protons would have exchanged with the deuterium oxide in the HPLC solvent. ^{1}H-NMR values from the literature for aaptamine (**1**) [49], isoaaptamine (**2**) [50], and aaptosine (**3**) [51] were compared to the experimental values obtained by LC–NMR (Table 1). The NMR data for aaptamine and isoaaptamine matched closely (average $\Delta\delta$=0.14 and 0.19 ppm, respectively) with the experimental results while there were significant discrepancies between aaptosine and the LC–NMR data (average $\Delta\delta$=0.89 ppm). Thus, aaptosine was eliminated, leaving aaptamine and isoaaptamine to be considered for the structure of the GFAT active component of *Aaptos* sp. While both aaptamine and isoaaptamine possess a methoxy group at C-8 (δ 4.03 and 4.02 ppm, respectively), aaptamine possesses a second methoxy group at C-7 (δ 3.86 ppm) and isoaaptamine bears the methyl group at N-1 (δ 4.09 ppm). Comparing these chemical shifts to the values from the LC–NMR experiment (Table 1), it is clear that the data for aaptamine fits the experimental data ($\Delta\delta$=0.04 for the two methyls), while the data for isaoaaptamine does not ($\Delta\delta$=0.16 for the two methyls). Thus, aaptamine (**1**) was concluded to be the structure of the active component of the dichloromethane extract of the

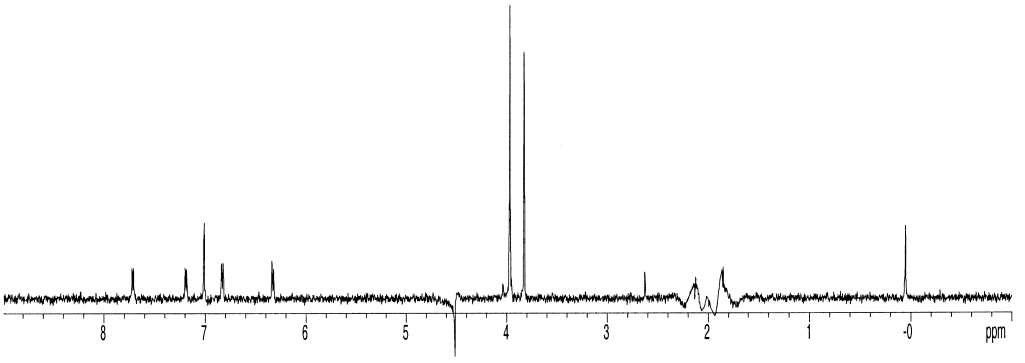

Fig. 3. ^{1}H-NMR (500 MHz) spectrum of the peak at 10.3 min in the crude extract of *Aaptos* sp. Data was acquired by stopped-flow LC–NMR using WET solvent suppression [46]. Disturbances in the baseline at 4.5 and 2.0 ppm are due to signals from residual water and acetonitrile.

Table 1
Comparison of ^{1}H-NMR data from the stopped-flow LC–NMR experiment on the peak at 10.3 min in the crude extract of the sponge *Aaptos* sp., aaptamine [49], isoaaptamine [50], and aaptosine [51]

Experimental[a]	Aaptamine (**1**)[b]	Isoaaptamine (**2**)[c]	Aaptosine (**3**)
7.72 (d, J=6.8 Hz)	7.90 (d, J=6.5 Hz, $\Delta\delta$=+0.18)	7.45 (d, $\Delta\delta$=0.27)	8.97 (d, J=5.9 Hz, $\Delta\delta$=+1.25)
7.19 (d, J=7.5 Hz)	7.45 (d, J=7.3 Hz, $\Delta\delta$=+0.26)	7.00 (d, $\Delta\delta$=0.19)	8.95 (d, J=4.8 Hz, $\Delta\delta$=+1.76)
7.01 (s)	7.18 (s, $\Delta\delta$=+0.17)	6.85 (s, $\Delta\delta$=0.16)	6.14 (s, $\Delta\delta$=+0.87)
6.83 (d, J=7.5 Hz)	6.93 (d, J=7.3 Hz, $\Delta\delta$=+0.10)	6.64 (d, $\Delta\delta$=0.19)	7.92 (d, J=5.9 Hz, $\Delta\delta$=+1.09)
6.33 (d, J=6.8 Hz)	6.52 (d, J=6.5 Hz, $\Delta\delta$=+0.19)	6.13 (d, $\Delta\delta$=0.20)	7.15 (d, J=4.8 Hz, $\Delta\delta$=0.82)
3.97 (s)	4.03 (s, $\Delta\delta$=+0.06)	4.09 (s, $\Delta\delta$=+0.12)	3.97 (s, $\Delta\delta$=0.00)
3.83 (s)	3.86 (s, $\Delta\delta$=+0.03)	4.02 (s, $\Delta\delta$=+0.19)	3.40 (s, $\Delta\delta$=0.43)

[a] Values in ppm (500 MHz). Solvent conditions approximately 43% ACN in D_2O (0.01% TFA).
[b] "$\Delta\delta$" represents the difference in chemical shift of comparable signals between experimental and literature values.
[c] Coupling constants for isoaaptamine were not reported [50].

sponge *Aaptos* sp. Purified aaptamine exhibited an IC_{50}=120 μM when assayed against GFAT.

4. Discussion

The dichloromethane extract of the marine sponge *Aaptos* sp. was found to inhibit the activity of GFAT in our HTS program. The marine alkaloid aaptamine (**1**) was determined to be responsible for the inhibitory activity using a combination of hyphenated analytical techniques. LC–MS was used to determine that the molecular mass of the active constituent was 228 u and LC–NMR provided the necessary structural information to confidently assign the structure of aaptamine. The combination of these two hyphenated analytical techniques provides an example of how LC–NMR can be incorporated into a natural products drug discovery program to increase the efficiency of the dereplication process.

NMR spectral data obtained using LC–NMR provides structural information which other hyphenated methods cannot. LC–NMR is especially useful in instances where the data from LC–MS is incomplete or does not allow the confident identification of the desired component of a sample. This provides a useful complement to the more sensitive and higher throughput methods of LC–MS and LC–DAD. Due to the limits in sensitivity and the lack of searchable NMR databases, LC–NMR is not yet ready for use as a front line dereplication technique. But, as we have demonstrated here, it can be used to augment the data obtained using more traditional dereplication techniques such as LC–MS and LC–DAD to allow for an efficient dereplication strategy. We recommend that LC–MS and/or LC–DAD methods continue to be used in their current primary roles for dereplication. The majority of samples can be confidently identified by these methods. But in the instances where the LC–MS and LC–DAD data provide numerous answers or when structural isomers are encountered, LC–NMR may provide the necessary data to complete the identification. It should be noted that the traditional natural products chemistry approach where compounds are isolated and structures are determined using NMR experiments in a tube will continue to be used. It is believed that for truly novel compounds, the traditional approach will still be the most efficient strategy.

At this time, the limited sensitivity of LC–NMR will prevent it from being as widely used of a hyphenated analytical technique as LC–MS is today, but LC–NMR provides a powerful tool for the rapid identification of known compounds and identification of structure classes of novel compounds. New strategies to increase the sensitivity of LC–NMR by trapping minor components on the chromatographic column and subsequent elution have been described [53,54]. In addition, technical advances in probe design and the production of higher field magnets will increase the sensitivity of this technique in coming years. Clearly, the power of LC–NMR has only begun to be recognized and the breadth of applications of this technique will grow dramatically in the future.

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