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Synthesis of Aspernigrin A

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Abstract

Aspernigrin A is a compound correlated with inhibiting the growth of cancer cells in the colon. A five-step synthesis was designed for aspernigrin A, projected to yield four intermediates and the final product. The intermediates were analyzed by high field ^{1}H-NMR and FT-IR spectroscopy before proceeding to the next step. A model pyridone ring system was successfully prepared from a commercially available pyrone precursor in two steps. These results provide a proof of concept that the synthetic scheme should successfully yield the natural product.

Introduction

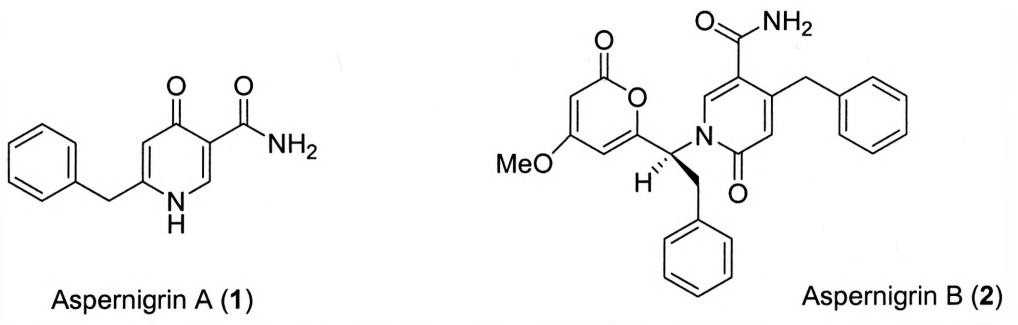


Figure 1. The structures of two compounds isolated from *Aspergillus niger*.

Cladosporium herbarum, IFB-E002 (isolate) is an endophytic fungus that lives in the leaves of *Cynodon dactylon* (crabgrass). Aspernigrin A, a biologically active metabolite, has been isolated from this fungus by Ye, et al[1]. This compound may inhibit the growth of line SW1116 of colon cancer cells[1]. Aspernigrin A (**1**, 4-benzyl-6-oxo-1,6-dihydropyridine-3-carboxamide, figure 1) has also been isolated by Hiort, et al from *Aspergillus niger,* which is a common fungus from the Mediterranean sea sponge, *Axinella damicornis*[2].

Aspergillus niger is a very common fungus; it is all around us. Right now we are breathing it in and out with no detrimental effects on our biological systems. Fungi have particularly interesting symbiotic relationships with their hosts. Generally, the relationship is mutually beneficial between the host and fungi[3]. This means that the fungus is provided a house to live in, while it gives the host some protection from predation[4]. To help protect their hosts from being eaten, many fungi excrete secondary metabolites. Aspernigrin A is a metabolite characterized as an alkaloid due to the fact that it contains nitrogen. Alkaloids often have an adverse effect on animals that would ingest them[5].

Aspernigrin B (**2**, figure 1) was also isolated from *Aspergillus niger*, the sea sponge symboint, and if the synthesis of aspernigirn A is successful, it may provide an important lead into methodology for the synthesis of aspernigrin B because of the structural similarities between the two compounds. Aspernigrin B is linked with protecting neuronal cell death caused by glutamic acid[2]. Thus aspernigrin B could be a lead compound for treatments for central nervous system diseases such as Alzheimer's.

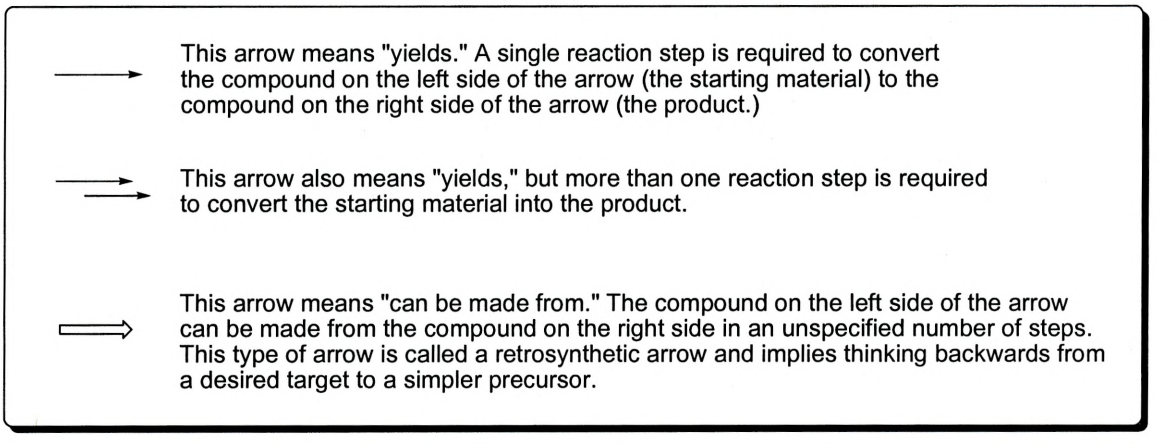
The observed biological activity shows these two compounds have the ability to react with receptors in the colon and brain that are possibly linked with color cancer and Alzheimer's disease, respectively. One receptor linked very closely with Alzheimer's disease is the NMDA (*N*-methyl D-aspartate) receptor[6]. This receptor is activated by elevated glutamic acid. Some glutamic acid is necessary in the brain because of its importance in synaptic plasticity or the ability of the connection between two neurons to change in strength, which is necessary in memory and learning. Elevated levels of glutamic acid can damage the cell membranes and trigger the mitochondria to break down, which releases apoptotic factors into the cell, which will eventually kill the cells. This neuronal death leads to damage in the frontal lobe.[7]

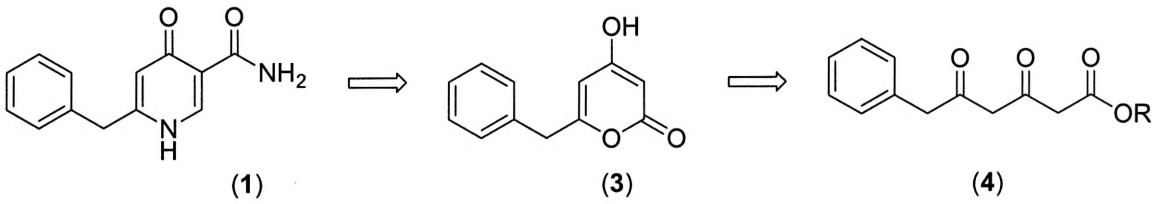
Due to its simpler structure, which lacks a stereocenter, we sought to synthesize aspernigrin A in a laboratory setting. Chemical synthesis of aspernigrin A is important because sea sponges are being harvested extensively and thus are becoming more scarce in the environment. Synthesis would prevent the over harvesting of sea sponges which would be necessary for isolation of this metabolite. Also, in the laboratory, more experiments can be performed to synthesize structural analogs, which may have increased bioactivity when compared to the bioactivity of aspernigrin A.

Achievement of this synthesis would allow for possible modifications to the laboratory synthesis making it more timely and effective by finding quicker ways to complete parts of the reaction or increase the yield. Cutting down the synthesis time would make it less costly and easier to reproduce. This could stimulate interest in synthesis and testing by pharmaceutical companies or cancer researchers. This could also lead researchers to delve deeper into the benefits of aspernigrin B and its synthesis due to the structural similarities between aspernigrin A and aspernigrin B.

Pharmaceutical companies may be interested in the aspernigrins if the compounds can be synthesized successfully and if they can be definitively linked to biological activity against cancer or Alzheimer's disease. Ultimately, a new drug could be developed for the treatment of cancer or Alzheimer's disease. If other diseases are associated with an excess of glutamic acid in the brain, treatment options may also be opened up for other central nervous system diseases.

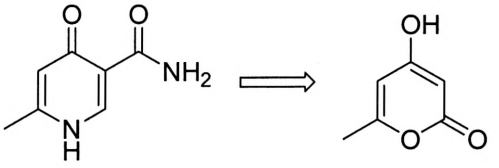
Results and Discussion





(1) (3) (4)

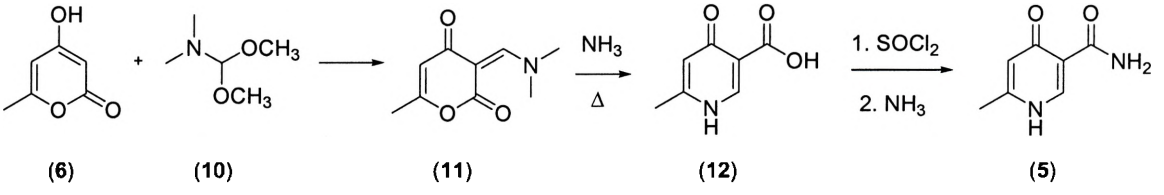
Scheme 1. Retrosynthetic analysis of aspernigrin A (**1**).



(5) (6)

Scheme 2. Model reactions for constructing the pyridone ring system.

Based on the examination of the literature,[8] we developed a retrosynthetic scheme, as seen in Scheme 1. The precursor of the pyridone ring of (**1**) is a pyrone (**3**), which can be obtained from the thermal cyclization of a diketoester (**4**) precursor. We then faced two challenges; the first was to make sure that the chemistry to convert the pyrone to the pyridone was possible. To develop the required reaction conditions, we chose to use commercially available 6-methylpyrone (**6**, scheme 2) as our model compound. Second, we had to construct the desired diketoester.[9] There are several ways to do this, however the main problem with this reaction stems from pKa issues relating to the α-H, which could result in very low percent yields, as described further below.



(6) (10) (11) (12) (5)

Scheme 3. Pyrone to pyridone reaction sequence.

The focus of my research project was the pyrone/pyridone model chemistry, shown in Scheme 3. The literature reference (Kilbourn & Seidel)[8] that was used as the basis for this chemistry was over 30 years old and complete data was not reported for all compounds. There have been many changes and advancements in technology since the 1970s. Because of this, it was necessary to perform a complete analysis of each intermediate. Not only was the reaction progress checked using TLC and purity assessed by melting point, but also FT-IR and FT-NMR data were used to confirm that the products had indeed been synthesized.

The addition reaction of dimethylformamide dimethylacetal (**10**) to 6-methylpyrone (**6**) in dioxane proceeded in 50% recrystallized yield. The melting point of (**11**) which was 146°C was close to the literature range of 152-154°C.[8] value and all spectroscopic data corresponded to the expected values. Once it was determined that (**11**) had indeed been formed, we could use the product of subsequent identical reactions after only TLC, melting point, and NMR analysis.

The second step required some method development. The literature reference called for ammonia gas to be bubbled through the reaction to isomerize pyrone (**11**) to pyridone (**12**).[8] For cost and safety reasons, however, we wanted to use methanolic ammonia. At first, we speculated that the reaction did not go to completion and an intermediate (3-(aminomethylene)-4-oxo-6-methyl-2-pyrone) (**13**) which according to Kilbourn & Seidel, was formed along with the desired product (**12**).[8] Because of this, we added 2-propanol to cause precipitation. Although the melting point of our product was higher than that reported for compound 13, we needed conclusive data that would help us assign the structure to differentiate between the two.

12 **13**

Figure 2. Compounds 12 and 13 represent the possible products of the pyridone isomerization reaction.

High field one- and two-dimensional NMR data was used to conclusively determine the structure of the intermediate because low-field one-dimensional NMR data were inconclusive. High field one-dimensional ^{1}H-NMR yielded the expected methyl and the two sp^2 hydrogen peaks; however, this provided no help in distinguishing between the two intermediates. The one-dimensional ^{13}C spectra showed the expected seven carbons at the appropriate chemical shifts as according to the literature.[8]

While this lead us to believe that we had actually made compound 12 (figure 2), we still needed to prove the absolute structure of the product based on chemical shift and correlations. To do this we used the nuclear Overhauser effect (NOE). NOE difference spectroscopy shows correlations through space as opposed to the traditional bond correlations. For the NOE experiment, (Figure 3, appendix) we chose a peak that we could assign unambiguously (methyl, 2.3 ppm) and irradiated it. As a result of the irradiation, a peak representing any hydrogen less than 4A away will appear as a positive (normal) peak after subtraction of the normal spectrum. Therefore, the peak that showed up in the positive direction is the peak representing the hydrogen that is next to the methyl group in space. In this case, the peak with a chemical shift of 6.5 ppm was the positive peak. Therefore, the H at 6.5 ppm is assigned to be the H next to the methyl

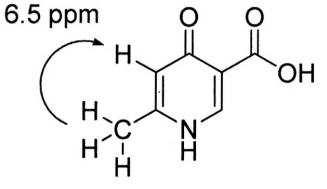


Figure 4. NMR #2 (NOE)

group. Spartan'02 data corroborates this conclusion with a calculated distance of 2.4A. This relationship, shown by Figure 4, also assigns the peak at δ=8.5 to the hydrogen next to the amine by default.

We also looked at an HSQC (heteronuclear single quantum correlation) spectrum. This spectrum correlates directly bound carbons and hydrogens (correlations through one bond). From this, we can figure out which hydrogen is directly connected to which carbon (Figure 5, appendix). We concluded that the H at 6.5 ppm (red) was directly correlated to the carbon at 117 ppm and the H at 8.5 ppm (green) was directly correlated to the carbon peak at 143 ppm. This left the last correlation of the methyl group at 2.3 ppm (blue) correlated with the carbon peak at 19.1 ppm.

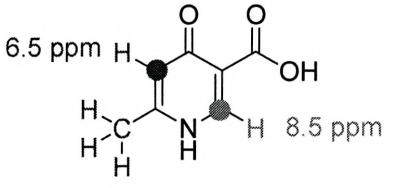


Figure 6. NMR #10 (HSQC)

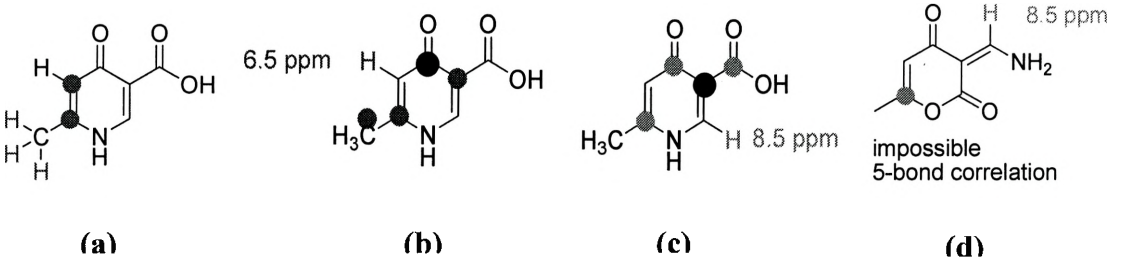


Figure 8. HMBC Correlations. Letters **a-c** represent correlations suggesting that compound 12 was the product and letter **d** shows why the product cannot be compound 13.

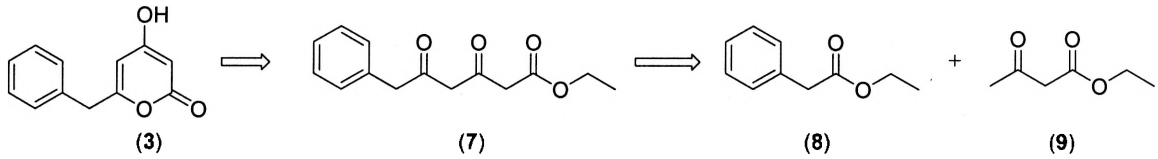
These correlations, however, do not positively differentiate desired compound 12 from 13. To do this we used an HMBC (heteronuclear multiple bond coherence). These spectra identify long range H-C correlations; that is, hydrogen to carbons two and three bonds away. The one-bond H-C correlations of the HSQC are not seen on this spectrum

(Figure 7, appendix). This allowed us to make the remaining carbon assignments, with the exception of the

carbonyls. These data eliminate the possibility of compound 13 being our product because the hydrogen at 8.5 ppm correlates to the carbon at 152 ppm. In compound 12, this carbon is three bonds away to the carbon at 152 ppm (Figure 8, **c**); however, in compound 13 it is five bonds away (Figure 8, **d**). This five-bond correlation would not show up on the HMBC. Also, when we compared our carbon assignments made using the spectral data to the literature values for aspernigrin A, our chemical shifts are nearly identical as seen in Table 1.[1]

Carbon	ppm
2	142
3	118
4	178
5	119
6	151
14	166

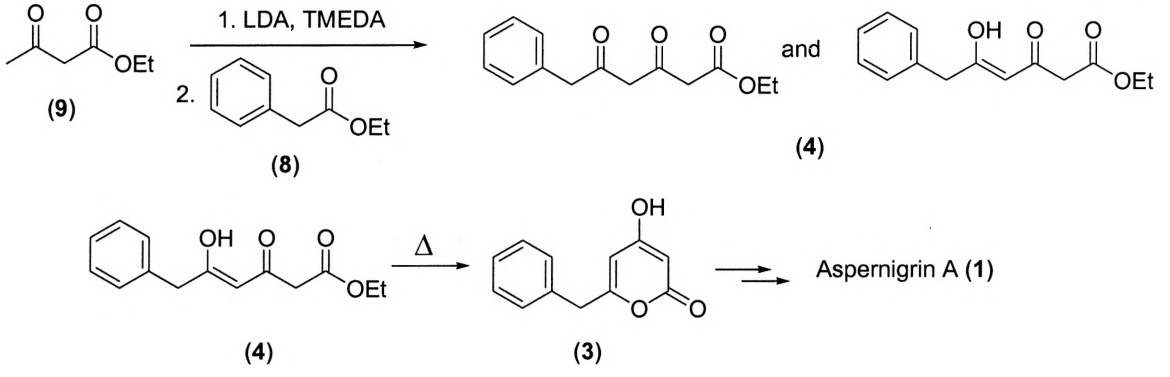
Table 1. NMR literature chemical shift data supporting the assignment of compound 12 as the product.[1]



Scheme 4. Retrosynthetic analysis of diketoester (**7**).

Now that we were confident that the isomerization reaction had been accomplished, a total synthesis of aspernigrin A (**1**) required benzylpyrone (**3**) as the precursor, as shown in Scheme 4, above. In the literature[9] the thermal cyclization of diketoesters and their equivalents has been used to yield pyrones. Thus, diketoester (**7**) was required. This synthesis, performed by Stephen Waters, was the second challenge we faced. To obtain (**7**), we should be able to acylate a dianion of ethyl acetoacetate (**9**, Scheme 5, below) with an ester or acid chloride of phenylacetatic acid. Because ethyl

phenylacetate (**8**) was readily available, we proceeded in this manner. This reaction was done three times, but no product was obtained until the last trial due to pKa issues with the α-hydrogen because it is unlikely that the reaction would proceed the way desired based n the pKa of the α-hydrogen. We tried to overcome this issue with very slow drop wise addition of the ethyl phenylacetate at -78°C; a very small amount of product (**7**) was isolated after column chromatography. High field NMR showed what we speculated to be a mixture of at least two enol tautomers. There are four possible enols of the diketo functionality and we were unable to determine the identity of our mixture from the NMR data. Molecular modeling using Spartan was used to determine which enol(s) were thermodynamically most stable. Because the heats of formation of the enols were within 1 kcal/mol of each other, we were again unable to see a significant difference.



Scheme 5. Synthesis of diketoester (**7**).

If more time for the project was allotted, further research would be to convert the carboxylic acid (**12**) made in the model reaction sequence to the amide (**5**) using thionyl chloride and ammonia. More research might also be done to optimize the isomerization with methanolic ammonia to improve the rate and chemical yield of the reaction. Additionally, the methyl pyridone (5) is a structural analog of aspernigrin A (1). A bioassay could also be developed to look into the bioactivity of aspernigrin A as well as

some of its structural analogs. It would also be beneficial to try using the synthetic aspernigrin A as a starting point to try to synthesize aspernigrin B.

Materials and Methods

General Methods. Melting points were obtained on a Mel-Temp II apparatus and are uncorrected. Thin layer chromatography employed aluminum backed Merck Silica Gel 60 plates with fluorescent indicator, which were cut to size. Infrared spectra were acquired on a ThermoNicolet Avatar 360 Fourier-transform infrared spectrometer using KBr pellets. Low field NMR spectra were obtained with an Anasazi Instruments, Inc., Eft-60 spectrometer interfaced to a Hitachi Perkin-Elmer R24B permanent magnet. High field 1H-, 13C-, and two-dimensional NMR spectra were recorded on a JEOL ECX 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Molecular modeling was conducted using Spartan'02, Wavefunction, Inc. Commercially available compounds and reagents were obtained from Aldrich Chemical Company.

3-(Dimethylaminomethylene)-4-oxo-6-methyl-2-pyrone (11). 4-Hydroxy-6-methyl-2-pyrone (**6**) (1.0049g, 7.98 mmol, 3.87% equiv.) was combined with 1.73 grams (14.8 mmol, 3.93% equiv) *N,N*-dimethylformamide dimethyl acetal (**10**) in 4 mL of p-dioxane and stirred until a brownish/red color appeared. Reaction progress was checked with thin layer chromatography (TLC) (5% methanol in chloroform). The reaction was capped and stored at 4°C overnight. 2-Propanol was added with stirring. The resulting solid was collected by vacuum filtration and crystallized from 2-propanol, yielding 0.6037 g, 50%, mp 146°C (literature[8] mp 152-154°C). ^{1}H-NMR (DMSO-d_6) δ = 2.17 (s,

3 H, C-methyl), 3.27 (s, 3 H, N-methyl), 3.52 (s, 3 H, N-methyl), 5.67 (s, 1 H, olefinic) and 8.32 (s, 1 H, olefinic) (Figure 9, appendix).

6-Methyl-4-(1H)-pyridone-3-carboxylic acid (12). 3-(Dimethylaminomethylene)-4-oxo-6-methyl-2-pyrone (**11**) (0.1205 g, 0.666 mmol, 0.463% equiv.) was combined with 1 mL of 2-methoxyethanol and 1 mL of 7 N methanolic ammonia in a 25 mL round bottom flask to form. The flask was capped and heated to a constant 47° C using a Thermowell® and sand bath. The resulting solution turned from colorless to red indicating progress in the reaction. Thin layer chromatography (5% methanol: chloroform) was run on the product and reactants. The sample was then rotovapped until only an oily residue remained and dissolved in 50 mL deionized water. The product was then acidified with 30 mL of glacial acetic acid, however no suspension formed. The sample was sealed and stored at 4°C overnight.

The sample was removed from the refrigerator and once it reached room temperature, toluene (20 mL) was added to lower the boiling point of the acetic acid and evaporated under low pressure. TLC (5% methanol in chloroform) was run and bromocresol green and ninhydrin stains were used. Bromocresol green was used to identify the presence of the carboxylic acid and the ninhydrin stain indicated the presence of an amine in the product. 2-Propanol was added and the sample was sealed and placed in an ice bath to encourage precipitation. The resulting solid was collected via vacuum filtration, collected and stored at 4°C. The filtrate was evaporated under low pressure, the oily residue that remained was sealed and stored at 4°C, yielding 0.0926g, 24%. Thin Layer Chromatography (5% methanol in chloroform) was run on both products, and the plates were stained with ninhydrin. Mp >225°C, IR (KBr) 3200 cm^{-1} (amine) (Figure 10,

appendix).^{1}H-NMR (DMSO-d_6) δ = 8.459 (C-H), 6.558 (C-H), 2.324 (methyl); NOE (irradiated methyl) δ=6.5; HSQC δ = 6.5 (117), 8.5 (143), 2.3 (19.1); HMBC: Carbon (δ) = 2 (143), 3 (114), 4 (179), 5 (119), 6 (152), 14 (167) (Figure 11, appendix).

Acknowledgements

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Figure 3 NOE difference spectra for 12

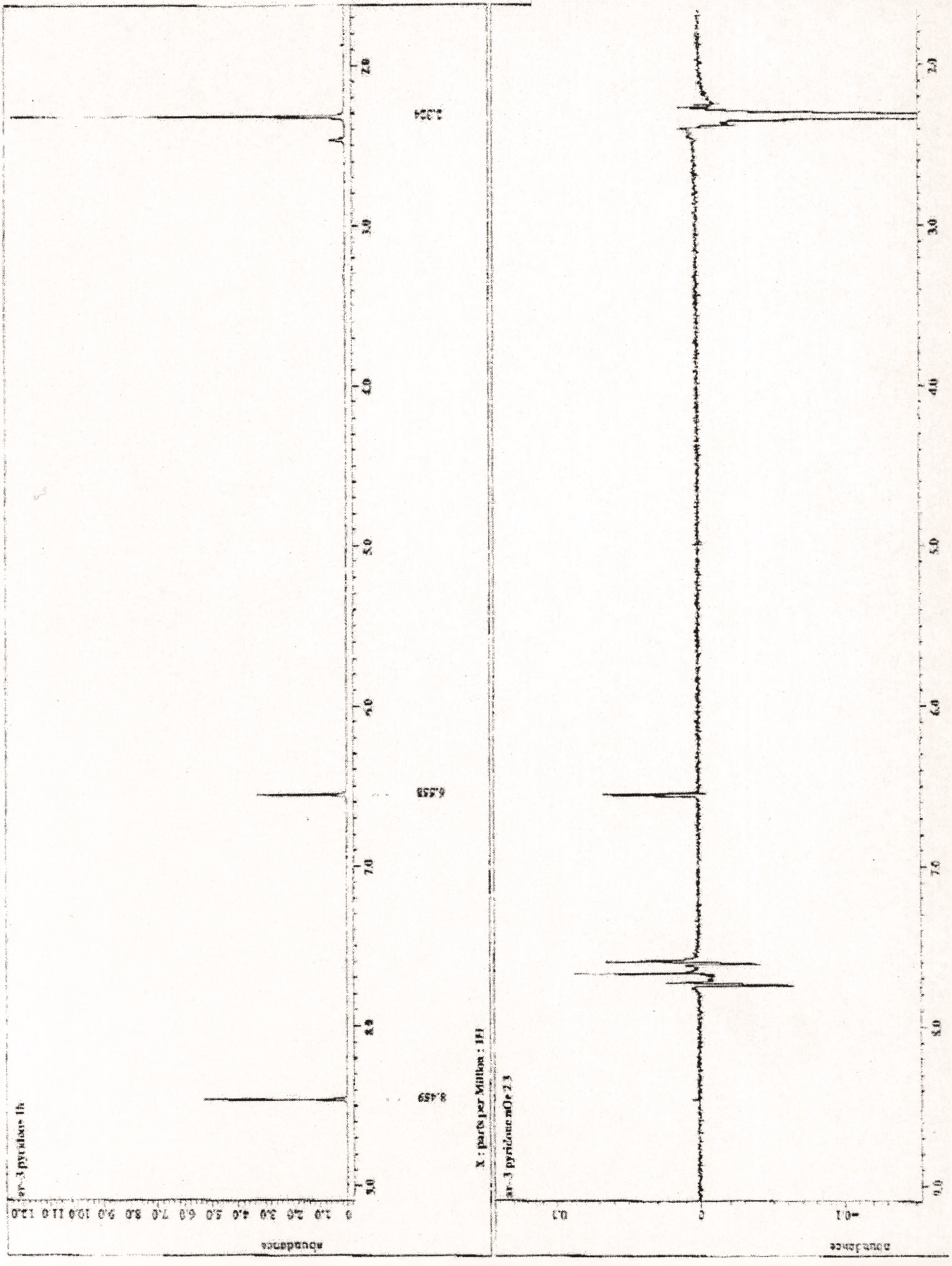


Figure 5 HSQC spectra for 12

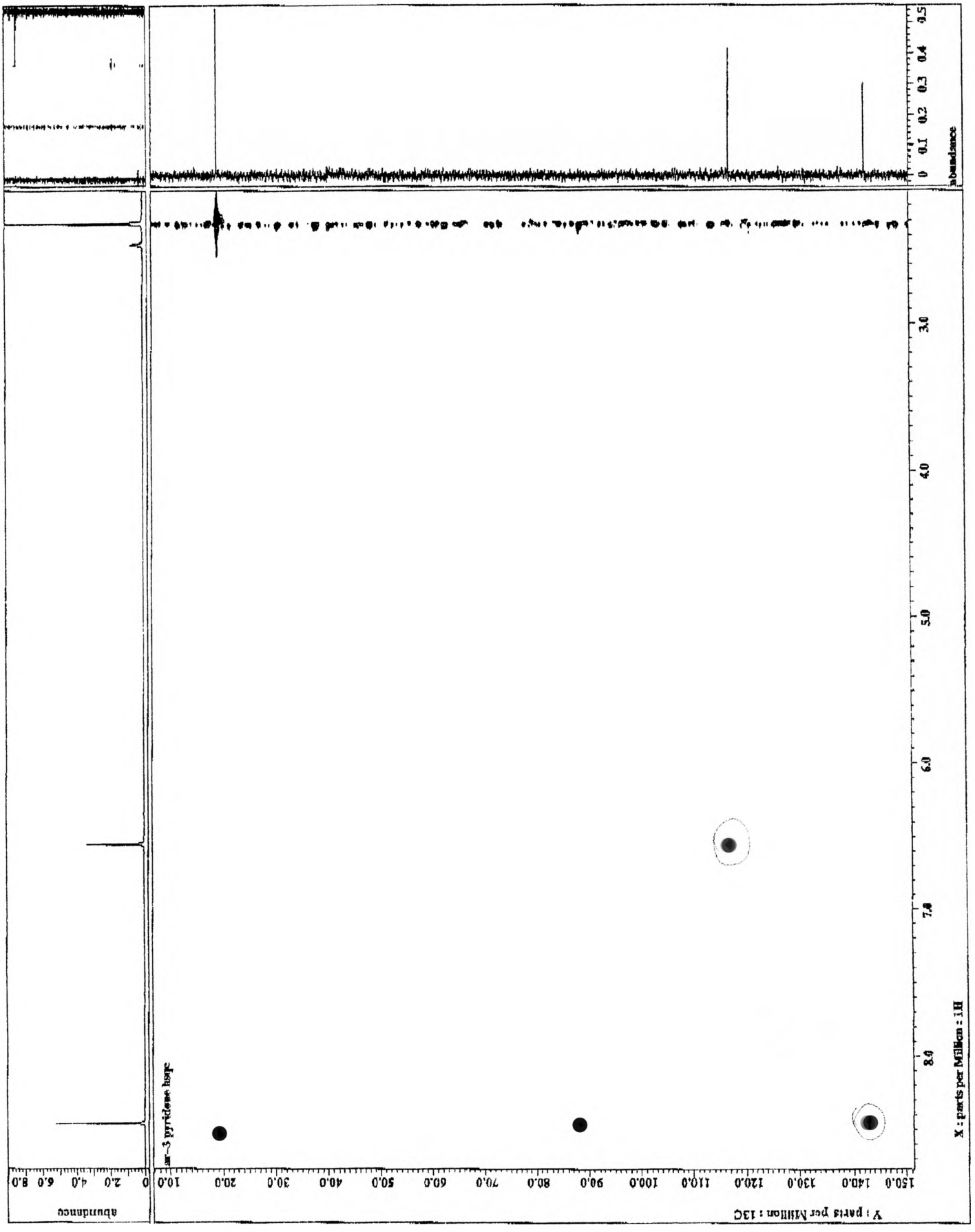
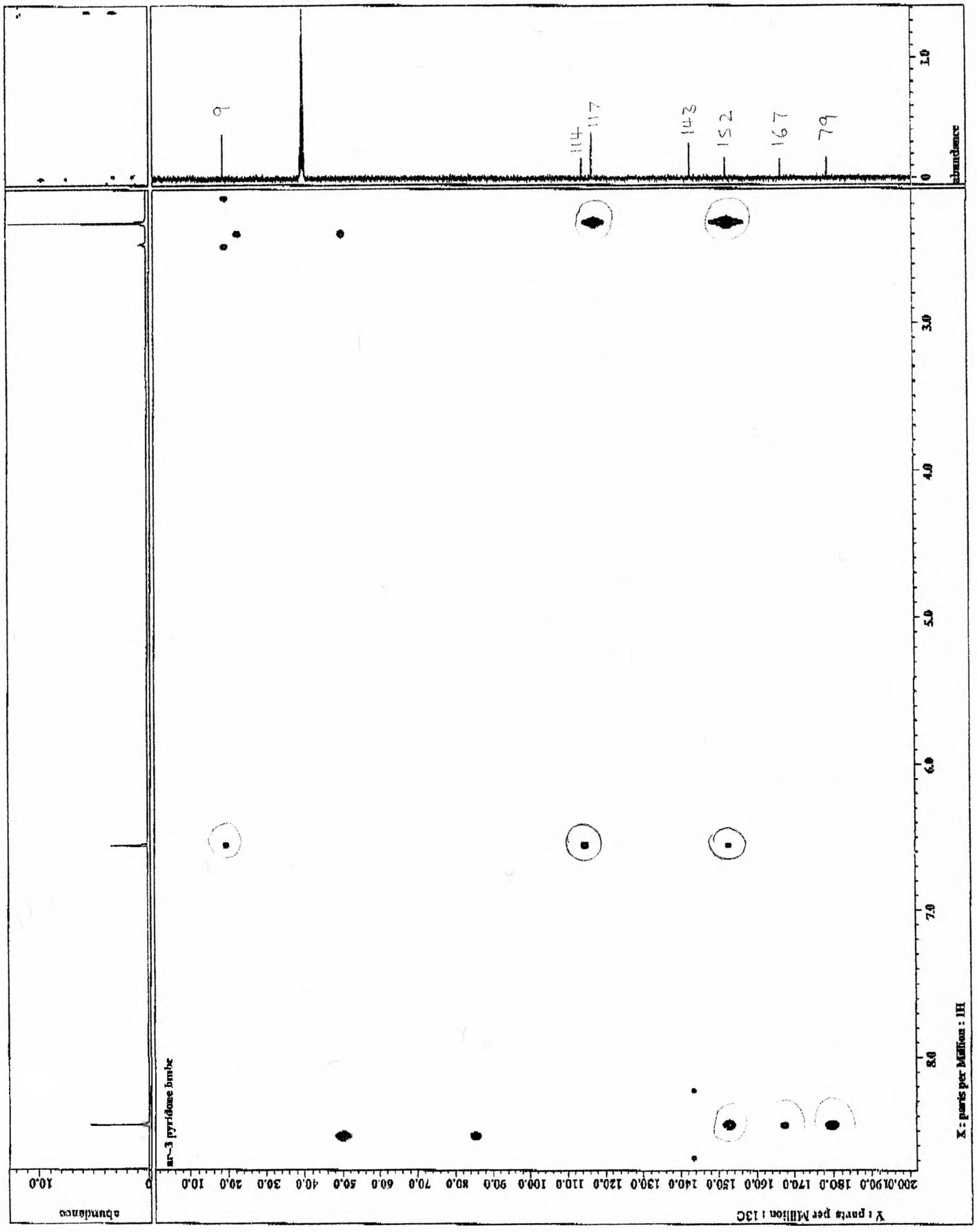


Figure 7 HMBC spectra for 12



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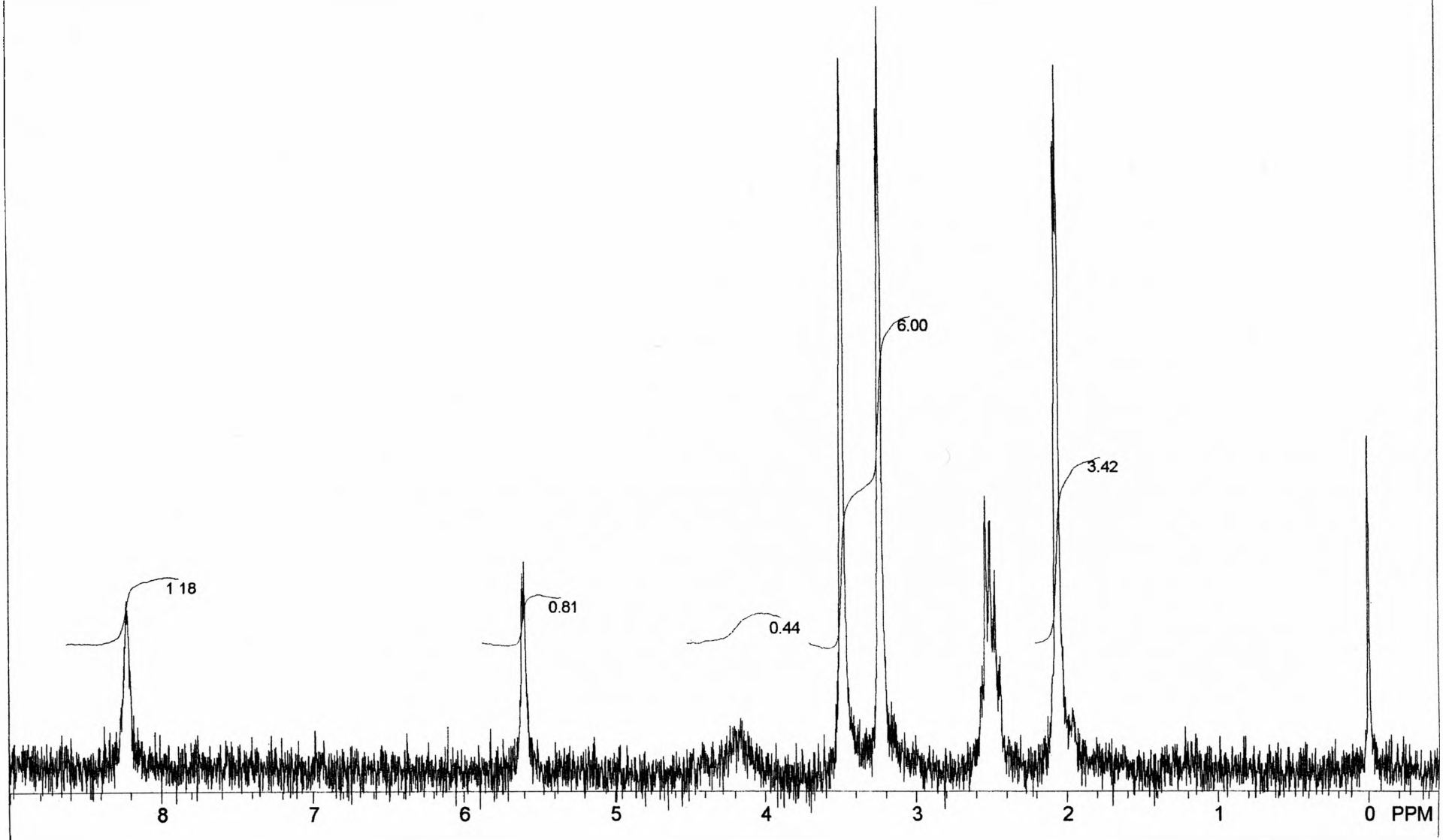

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Figure 9 ^{1}H-NMR for 11

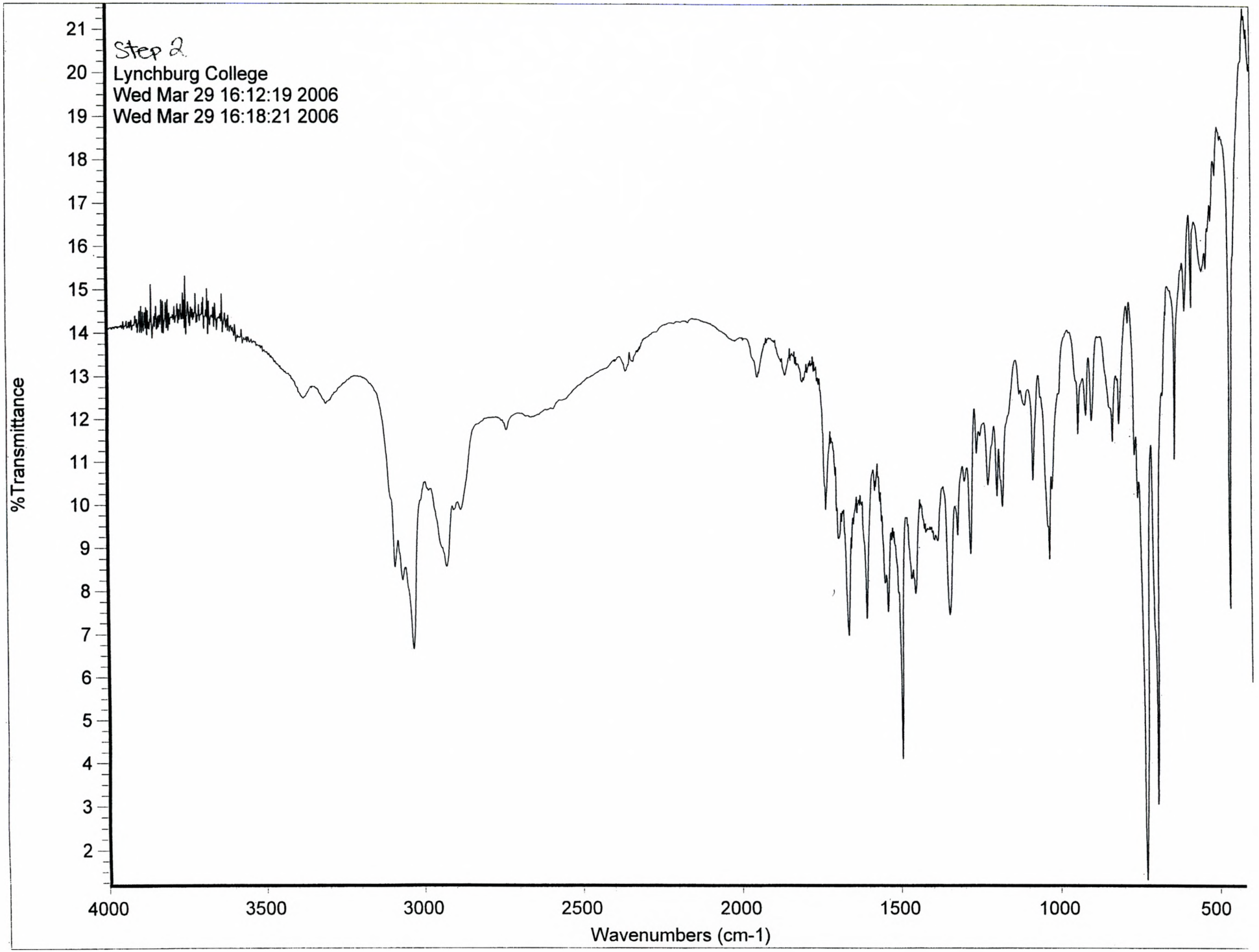

Figure 10 IR for 12

Figure 11 ¹H-NMR for 12