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Preparation of Stable Isotope-Labeled 2-Nitrobenzaldehyde Derivatives of Four Metabolites of Nitrofuran Antibiotics and Their Comprehensive Characterization by UV, MS, and NMR Techniques

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A convenient method is presented for the preparation of the carbon-13-labeled 2-nitrobenzaldehyde derivatives of the nitrofuran metabolites 3-amino-2-oxazolidinone (AOZ), semicarbazide (SC), 1-aminohydantoin (AH), and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), with the purpose of using them as internal standards for the quantification of trace levels of nitrofuran residues by liquid chromatography−tandem mass spectrometry in foods of animal origin. The synthesis encompasses the nitration of [1,2,3,4,5,6-$^{13}C_6$]toluene prior to chromyl compound-mediated oxidation of the methyl group into the corresponding aldehyde. The four metabolites of nitrofuran antibiotics were derivatized independently with the resulting ring-labeled 2-nitrobenzaldehyde (NBA) to obtain the target compounds. Both the isotopically enriched and native substances were used to perform a comprehensive fragmentation study by electrospray ionization (ESI) collision-induced dissociation (CID) mass spectrometry (MS). Full characterization of the nitrofuran derivatives was accomplished with ultraviolet (UV) and exhaustive nuclear magnetic resonance (NMR) analysis. A major advantage of the described procedure is that it can be extended to the preparation of other carbon-13-labeled derivatives of metabolites of nitrofuran antibiotics.

KEYWORDS: Stable isotope; nitrofuran antibiotics; metabolites; 3-amino-2-oxazolidinone; semicarbazide; 1-aminohydantoin; 3-amino-5-morpholinomethyl-2-oxazolidinone; synthesis; NMR; MS

INTRODUCTION

Some nitrofuran derivatives are synthetic chemotherapeutic agents with a broad antimicrobial spectrum. They are active against both Gram-positive and Gram-negative bacteria, including species belonging to the genera *Salmonella* and *Giardia*, trichomonads, amebae, and some coccidial species. Nitrofurans have the advantage that in vivo bacterial resistance appears to develop slowly and to a limited degree (*1*, *2*). The most common of these are furazolidone [*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone], nitrofurantoin [*N*-(5-nitro-2-furfurylidene)-1-aminohydantoin], nitrofurazone (5-nitro-2-furaldehyde semicarbazone), and furaltadone (furazolidone with a morpholinomethyl group bound to the oxazolidinone ring). Nitrofurans are administered to chickens, turkeys, swine, and cows to treat diseases such as digestive and urinary tract infections.

Nitrofuran-type drugs are metabolized rapidly in vivo, leading to a significant decrease in plasma levels of the parent compound within a short time frame of several minutes to a few hours (*3*, *4*). A concomitant accumulation of some metabolites in proteins is observed, generating stable adducts that can be detected in tissues over periods of up to 30 days (*4*−*7*). Therefore, metabolites of nitrofuran antibiotics can occur in meat from treated animals or in animals fed residue-contaminated food (*8*). Due to the potential harmful effects of these metabolites, the European Union (EU) has prohibited the use of nitrofuran antibiotics in food-producing animals (*9*). In addition, the recent detection of metabolites of nitrofuran antibiotics in poultry and shellfish imported into the EU (*10*) warrants the development of analytical methods that combine selectivity, accuracy, and sensitivity to reach the minimum required performance limit (MRPL) set at 1 μg/kg (*11*) for the determination of the residues of these banned products in different commodities.

The analytical methods reported in the literature to determine nitrofurans are based on the acid-catalyzed release of protein-bound metabolites, followed by their conversion into the 2-nitrobenzaldehyde (NBA) imine-type derivatives. These can be detected by either ultraviolet (UV) (*12*, *13*) or mass spectrometry (MS)-based methods (*14*, *15*). The major drawback of high-performance liquid chromatography−ultraviolet (HPLC-UV)-based techniques is their lack of sensitivity, making MS and particularly tandem MS attractive alternatives to confirm the occurrence of any nitrofuran residues in foods of animal origin. Thermospray mass spectrometry (TSP-MS) was em-

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ployed in the single ion monitoring mode to quantify 3-amino-2-oxazolidinone (AOZ) in porcine tissues (*14*), and both AOZ and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) were determined simultaneously by using a single quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) interface (*12*). In liver, a limit of detection at 10 ng/g was achieved for both metabolites. Recently, a liquid chromatography−tandem mass spectrometry (LC-MS/MS) method was developed to analyze the four metabolites of nitrofuran antibiotics as their NBA derivatives in the same analytical run with limits of detection ranging from 0.5 to 5 ng/g of muscle tissue (*15*). The latter method employs an internal standard approach by supplementing samples with the 4-nitrobenzaldehyde derivative of semicarbazide to improve the quantitative aspect of the method. However, the signal suppression effect observed commonly by electrospray (*16*, *17*) may be retention time-dependent and may lead to inaccurate quantification even if an analogue product is used as an internal standard. Furthermore, this effect could even mask the detection of some confirmatory transitions (qualifier ions), leading to ambiguous or erroneous conclusions. Therefore, the best approach is the use of stable isotope-labeled internal standards, which provides added certainty and confidence in the detection of the analytes in complex food matrices. A recent study showed that HPLC-UV and LC-MS/MS methods provide comparable results with regard to the level of tissue-bound AOZ within the range of 200−1000 μg/kg (*18*).

In the present work, a convenient synthesis to prepare carbon-13 ring-labeled NBA of four important nitrofuran derivatives is described. This approach has the advantage that it can easily be extended to the preparation of other derivatives of nitrofuran-type antibiotics. The synthesis encompasses a nitronium tetrafluoroborate-mediated nitration of the carbon-13 ring-labeled toluene prior to conversion of the methyl group into the benzaldehyde with chromium trioxide. The four carbon-13-labeled internal standards are subsequently obtained by the derivatization of the metabolite in the presence of carbon-13-labeled NBA. The UV data, a comprehensive MS fragmentation study, and the complete assignment of the hydrogen and carbon atoms by exhaustive NMR techniques of the four metabolite derivatives are presented.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile, acetic anhydride, dichloromethane, hexane, sulfuric acid, and sodium sulfate were supplied by Merck (Darmstadt, Germany). NBA and chromium trioxide (CrO_3) were obtained from Sigma-Aldrich (Steinheim, Germany), and nitronium tetrafluoroborate (NO_2BF_4) was from Aldrich (Milwaukee, WI). [1,2,3,4,5,6-$^{13}C_6$]Toluene (>97 atom % ^{13}C) was purchased by Cambridge Isotope Laboratories (Andover, MA). AOZ, 1-aminohydantoin (AH) (hydrochloride form), and semicarbazide (SC) (hydrochloride form) were obtained from Aldrich. AMOZ was a kind gift of Dr. L. A. P. Hoogenboom (Rikilt, The Netherlands). DMSO-d_6 (99.95 atom % D) and tetramethylsilane (TMS) were obtained from Dr. Glaser A.G. (Basel, Switzerland). Unlabeled NBA derivatives of 3-amino-2-oxazolidinone (NBAOZ), semicarbazide (NBSC), 1-aminohydantoin (NBAH), and 3-amino-5-morpholinomethyl-2-oxazolidinone (NBAMOZ) were purchased from Witega (Berlin, Germany).

Synthesis of Carbon-13-Labeled NBA. Three hundred microliters (2.83 mmol) of [1,2,3,4,5,6-$^{13}C_6$]toluene was mixed with 6 mL of acetonitrile, and the resulting solution was kept under continuous stirring at room temperature. Then, 413 mg of NO_2BF_4 (3.11 mmol, 1.1 equiv) was added, and the mixture was kept overnight at room temperature under stirring. Acetonitrile was removed by distillation, and the oily resulting residue was suspended in dichloromethane (15 mL). The organic solution was washed two times with water (each 15 mL) and dried over Na_2SO_4. Dichloromethane was evaporated under a gentle stream of nitrogen, and the residue was dissolved in 10 mL of acetic anhydride. The resulting solution was kept at 2 °C in an ice bath. Then, 2 mL of sulfuric acid was slowly added to the mixture, keeping its temperature below 5 °C. Chromium trioxide (849 mg, 8.49 mmol, 3.0 equiv) suspended in acetic anhydride (5 mL) was then slowly added to the reaction mixture (temperature maintained below 10 °C). The solution was stirred gently for 2 h, and the temperature set at 2 °C prior to the addition of 40 mL of cold water with vigorous stirring. Both [1,2,3,4,5,6-$^{13}C_6$]-2-nitrobenzaldehyde ([$^{13}C_6$]NBA) and [1,2,3,4,5,6-$^{13}C_6$]-4-nitrobenzaldehyde were extracted simultaneously with dichloromethane, and the resulting organic solution was dried over Na_2SO_4. The dichloromethane solution was evaporated to dryness under a gentle stream of nitrogen, and the residue was reconstituted in hexane. The organic solution was loaded onto an open air column containing 2.5 mg of silica gel Chromabond SiOH (Macherey-Nagel, Düren, Germany), and [$^{13}C_6$]NBA was purified with a step gradient of dichloromethane (0−7%, step = 0.5%). The appropriate 5 mL fractions were pooled, affording ~35 mg of [$^{13}C_6$]NBA (232 μmol, yield = 8.2%). The conversion of [1,2,3,4,5,6-$^{13}C_6$]nitrotoluene into [$^{13}C_6$]NBA was controlled by thin-layer chromatography (TLC) using silica gel 60 F_{254} aluminum sheets (Merck) with a mixture of hexane/dichloromethane 25:75 (v/v) as an elution solvent. The R_f values were 0.47 and 0.68 for NBA and 4-nitrobenzaldehyde, respectively.

Derivatization of Nitrofuran Metabolites with Carbon-13-Labeled NBA. *Derivatives of AOZ, AH, and AMOZ.* Approximately 4.4 mg of [$^{13}C_6$]NBA (28 μmol) dissolved in a mixture of hexane/dichloromethane was evaporated to dryness prior to reconstitution in 150 μL of DMSO. The solution was then further diluted in 20 mL of 0.125 M hydrochloric acid. Three molar equivalents of either AOZ, AMOZ, or AH was added, and the resulting solutions were kept at room temperature overnight. After neutralization with both 1 M sodium hydroxide and 1 M dipotassium hydrogen phosphate, the compounds of interest were extracted three times with 10 mL of ethyl acetate. The resulting organic phase was concentrated to dryness and rediluted in distilled water/acetonitrile (70:30, v/v) before purification by HPLC.

Derivative of SC. Approximately 4.4 mg of [$^{13}C_6$]NBA (28 μmol) dissolved in a mixture of hexane/dichloromethane was evaporated to dryness prior to reconstitution in 10 mL of 1.2 M sodium acetate/DMSO (95:5, v/v). Three molar equivalents of SC was added, the solution was stored overnight at room temperature, and the compound of interest was extracted three times with 10 mL of ethyl acetate. The resulting organic phase was concentrated to dryness in vacuo and rediluted in water/acetonitrile (70:30, v/v) prior to HPLC purification.

HPLC Purification. Purifications were carried out on a SymmetryPrep C18 7 μm column (7.8 × 150 mm) from Waters (Milford, MA). The mobile phase was constituted of distilled water (solvent A) and acetonitrile (solvent B). The gradient was as follows: 0−1 min at 10% B (3 mL/min); 1−10 min from 10 to 30% B (3 mL/min); 10−13 min at 30% B (3 mL/min); 13−14 min from 30 to 100% B (from 3 to 5 mL/min); 14−18 min at 100% B (5 mL/min); 18−19 min from 100 to 10% B (5 mL/min); and 19−25 min at 10% B (5 mL/min). The detection wavelength was 260 nm. Retention times were as follows: NBSC, 11.1 min; NBAH, 11.9 min; NBAOZ, 13.1 min; NBAMOZ, 13.9 min. The products were examined by UV and ^1H and ^{13}C nuclear magnetic resonance (NMR) as well as collision-induced dissociation (CID) MS.

UV Spectroscopy. The UV absorption spectra were obtained in pure water (pH 5.9) on a Lambda Bio 20 UV−visible spectrophotometer (Perkin-Elmer, Norwalk, CT) controlled by the UV WinLab data processing system (Perkin-Elmer). Spectra were recorded within the wavelength range of 220−420 nm, at a scan speed of 60 nm/min. Extinction coefficients were obtained from UV data of solutions of unlabeled derivatives of metabolites of nitrofuran antibiotics (10 mg first dissolved in 5 mL of methanol and further diluted in water).

MS. ESI-CID-MS spectra were recorded in the positive mode on a Quattro LC (Micromass, Manchester, U.K.). The capillary voltage was set at 3.0 kV and the radio frequency lens at 0.20 V. Source block and desolvation temperatures were 100 and 350 °C, respectively. Nitrogen gas was used for nebulization and desolvation with flow rates at 60 and 400 L/h, respectively. LM (low mass) and HM (high mass)

resolutions were both set at 15.0 on quadrupoles 1 and 2. Argon was used as a collision gas, and the pressure in the collision cell was set at 2.7 mTorr. The fragmentation pathways were confirmed by performing multiple-step CID on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray interface. The source and capillary voltages were 4.0 kV and 36 V, respectively. The capillary temperature was set at 250 °C, and the sheath and auxiliary gases were at 86 and 48 arbitrary units, respectively. Samples were dissolved in a mixture of acetonitrile/water to reach a concentration of 10 μg/mL approximately, and infusions were performed with a flow rate ranging from 5 to 10 μL/min.

NMR Spectroscopy. The samples (0.5−4 mg) for NMR spectroscopy were prepared in Wilmad 528-PP 5 mm Pyrex tubes, using ∼0.75 mL of DMSO-d_6 as solvent from a sealed vial. The NMR spectra were recorded on a Bruker DPX-360 spectrometer, equipped with a broadband multinuclear *z*-gradient probehead, at 360.13 MHz for proton and at 90.56 MHz for carbon-13. The probe temperature was 21 °C for the proton spectra recording and slightly higher for the carbon spectra, due to heteronuclear composite pulse decoupling. The chemical shifts (in parts per million) were expressed with respect to tetramethylsilane (TMS) as a reference. The assignment of the proton signals was based on the results of the COSY (two-dimensional homonuclear shift correlation with gradient selection) and COSY long-range. Homonuclear nuclear Overhauser effect (NOE) difference spectroscopy was used for establishing the stereochemistry of the metabolite derivatives. For carbon atoms, the assignments were carried out with data from DEPT 135 spectra and HSQC experiment (two-dimensional inverse detected heteronuclear direct bond shift correlation with gradient selection) optimized for coupling constants at 145 Hz. Additional information was obtained from the HMBC experiment (two-dimensional inverse detected heteronuclear long-range shift correlation with gradient selection) optimized for coupling constants at 8 Hz. The ^{13}C NMR spectra were simulated with ChemWindow 6.5 spectroscopy software (Microsoft).

RESULTS AND DISCUSSION

The goal of this study was to develop a synthetic route to prepare [$^{13}C_6$]NBA, which could serve as a derivatizing agent for several metabolites of nitrofuran antibiotics, thereby circumventing tedious synthesis of the individual isotope-enriched compounds (e.g., AOZ, AH, SC, and AMOZ). Indeed, tetradeuterated AOZ and pentadeuterated AMOZ as well as carbon-13- and nitrogen-15-labeled SC are now commercially available, but no stable isotope-labeled AH exists so far. In addition, our preliminary studies revealed a degradation of AOZ, AMOZ, and AH during the hydrochloric acid-mediated hydrolysis. These results suggest that the supplementation of nonderivatized stable isotope-labeled compound prior to the acidic treatment could lead to an overestimation of the level of tissue-bound AOZ, AMOZ, and AH. Hence, our approach allows the preparation of the carbon-13-labeled 2-nitrobenzaldehyde derivatives of the four metabolites of AOZ, AH, SC, and AMOZ, and their posthydrolysis addition in the sample will provide an accurate and precise quantification of the released metabolites in the extract.

Synthesis of [$^{13}C_6$]NBA. We planned to perform a nitration of [1,2,3,4,5,6-$^{13}C_6$]toluene prior to a chromyl compound-induced oxidation of the methyl group affording [$^{13}C_6$]NBA. Our initial attempt to nitrate carbon-13-labeled toluene with tetranitromethane was unsuccessful due to some solvent incompatibility. The tetranitromethane-mediated nitration of toluene has to be conducted in a protic solvent, whereas the conversion of the methyl group into the aldehyde requires neither water nor alcohol. If present, these solvents would prevent the formation of the complex between the methyl group of toluene and the chromyl compound (*19*). Therefore, we used nitronium tetrafluoroborate in acetonitrile to prepare [$^{13}C_6$]NBA by employing [1,2,3,4,5,6-$^{13}C_6$]toluene as the starting material (*20*).

We first tried to convert the methyl group of [1,2,3,4,5,6-$^{13}C_6$]toluene into the aldehyde by applying the Etard reaction. This reaction involves the addition of chromyl chloride, followed by a hydrolysis step to yield aldehydes, ketones, and quinones (*19*). Unfortunately, this approach led to a very low yield in the case of 2-nitrotoluene. Indeed, the electron-rich nitro moiety weakens the bond of the complex and changes the electron pattern so that the addition product forms with difficulty, leading to the regeneration of the original compound during hydrolysis (*19*, *21*). We therefore decided to employ chromium trioxide as the oxidizing reagent (*22*). The oxidation was conducted in acetic anhydride in the presence of concentrated sulfuric acid, maintaining the temperature below 10 °C. Under such conditions, the conversion of the methyl group into the corresponding aldehyde occurred with a satisfactory yield (∼50% based on TLC analysis). However, the reaction mixture still contained some unreacted [1,2,3,4,5,6-$^{13}C_6$]nitrotoluene as well as polar products, presumably [1,2,3,4,5,6-$^{13}C_6$]nitrobenzoic acids.

The main drawback of the reaction was the formation of [1,2,3,4,5,6-$^{13}C_6$]-4-nitrotoluene as a byproduct generated during the nitration of [1,2,3,4,5,6-$^{13}C_6$]toluene. It is well-known that the para position is much more favorable to electrophilic substitutions than the ortho carbon, particularly in the case of a nitronium ion as a substitution reagent. Consequently, the yield of [$^{13}C_6$]NBA from [1,2,3,4,5,6-$^{13}C_6$]toluene did not exceed 10%.

UV Spectra. As anticipated, the UV spectra of NBAOZ, NBSC, NBAH, and NBAMOZ are comparable (**Figure 1**) in that they all exhibit a maximum of absorption at 255−265 nm and a shoulder at 310−340 nm. The molar extinction coefficients (L·mol^{-1}·cm^{-1}) were determined as follows: NBAOZ (259 nm), 16305; NBSC (263 nm), 15477; NBAH (264 nm), 18396; NBAMOZ (256 nm), 17723.

Fragmentation by ESI-CID-MS. *NBAOZ.* Tandem MS experiments were performed on either NBAOZ or [$^{13}C_6$]-NBAOZ to obtain valuable information regarding the fragmentation pathway of the NBA derivative of AOZ. The full scan spectrum of NBAOZ ([$^{13}C_6$]NBAOZ, respectively) exhibited a prominent ion at *m*/*z* 236 (*m*/*z* 242, respectively) corresponding to the protonated molecule $[M + H]^+$. The 15 eV product ion mode spectrum of NBAOZ (*m*/*z* 236 as the precursor ion) exhibited a base peak at *m*/*z* 134 ($\Delta M = -102$ Da) with two additional higher mass fragment ions at *m*/*z* 149 ($\Delta M = -87$ Da) and 192 ($\Delta M = -44$ Da) (**Figure 2**). Fragment ions at *m*/*z* 134 and 192 were observed in the MS^2 spectrum recorded with the ion trap analyzer, whereas ion *m*/*z* 149 was present only in the MS^3 spectrum when the acquisition mode was *m*/*z* 236 → *m*/*z* 192 → *m*/*z* 50−250. This clearly indicates that the ion at *m*/*z* 149 is generated by the fragmentation of the ion at *m*/*z* 192. The loss of 44 Da can be rationalized in terms of the elimination of carbon dioxide from the oxazolidinone moiety, giving rise to the ion at *m*/*z* 192. A subsequent hydrogen abstraction by an oxygen atom of the nitro group would lead to the elimination of HN=$CHCH_3$, generating the fragment ion at *m*/*z* 149. This hydrogen transfer process has already been observed by electron impact with *N*-substituted *o*-nitroanilines (*23*) and heterocyclic nitramines (*24*). The formation of the ion at *m*/*z* 134 can be explained by a nitro group oxygen-mediated nucleophilic attack on the carbon in the α-position of the aromatic ring, generating a transient species with a five-membered ring involving atoms C-1, C-2, NO-2, NO-2, and the carbon in the α-position of the aromatic ring. A subsequent cleavage of the N−O bond would promote an electronic

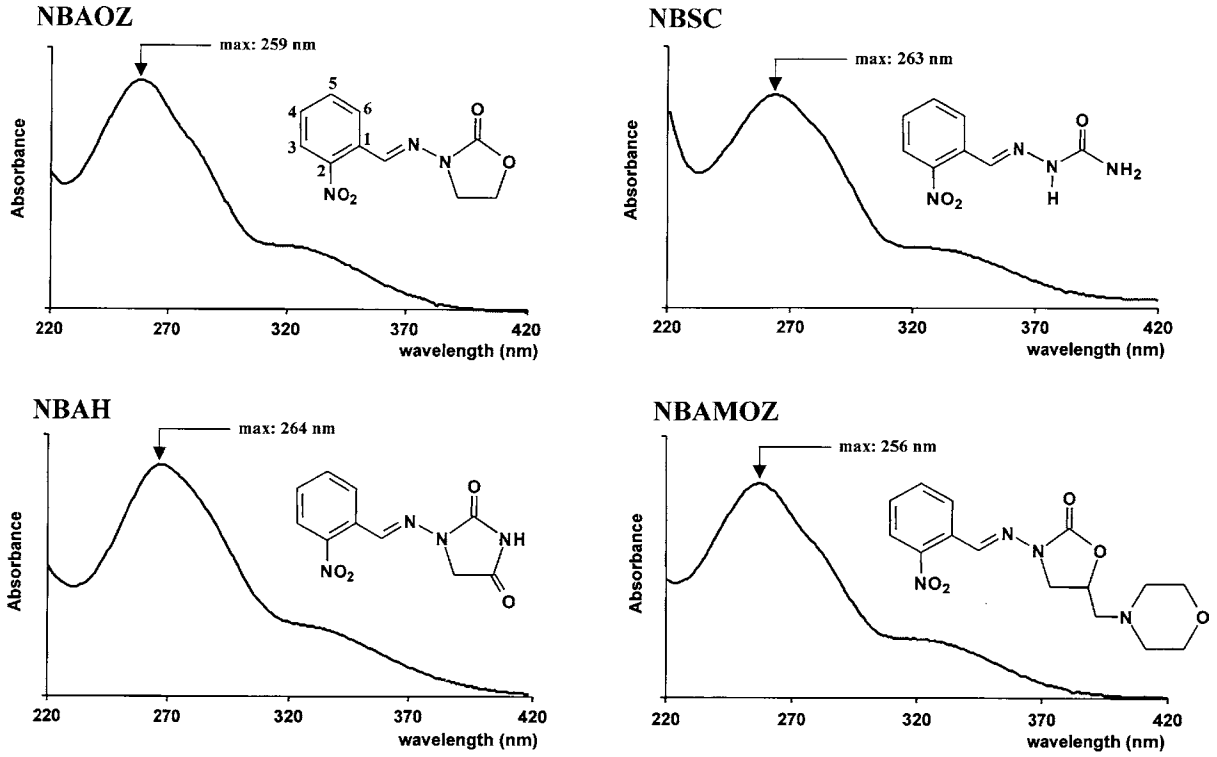

Figure 1. UV spectra of NBAOZ, NBSC, NBAH, and NBAMOZ (in water, pH 5.9).

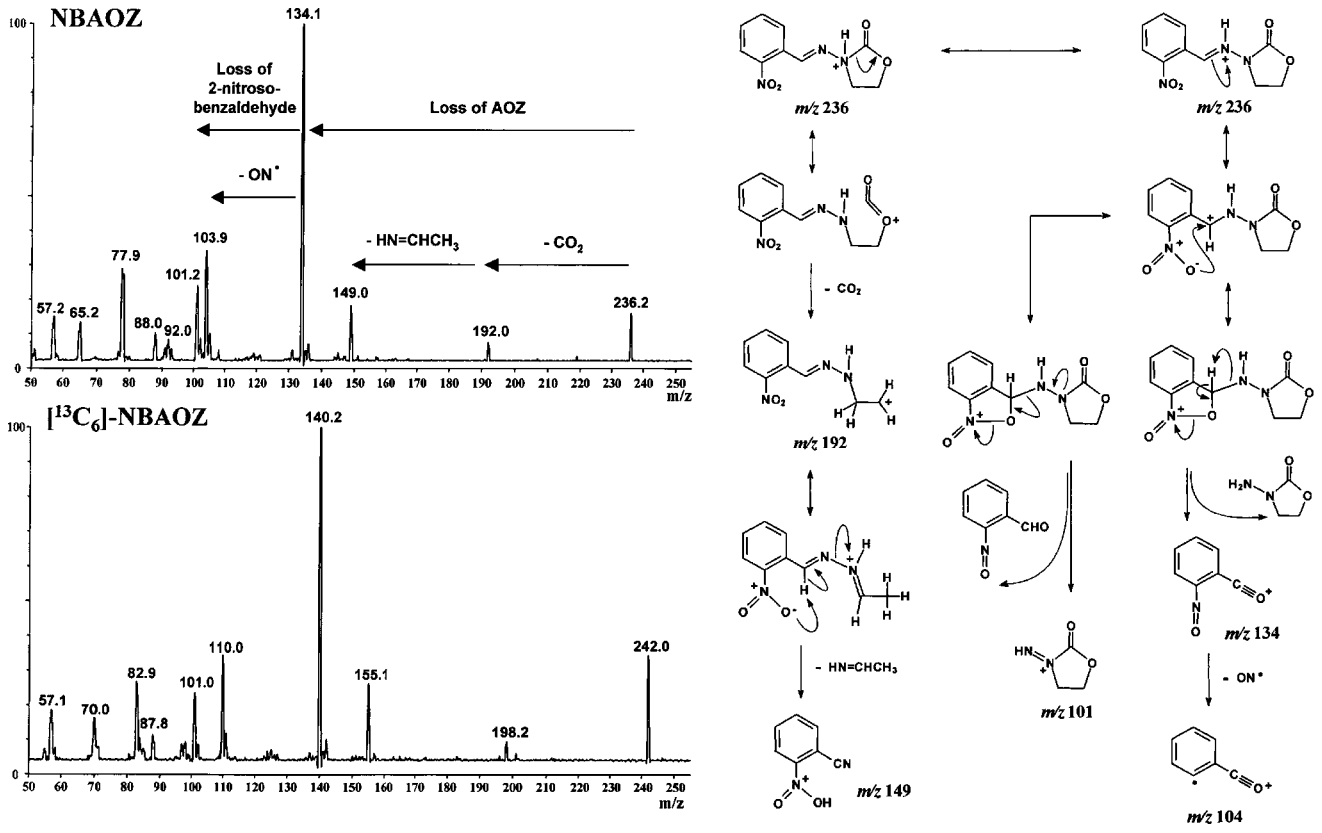

Figure 2. CID spectra of both NBAOZ and [$^{13}C_6$]NBAOZ, recorded at 15 eV, and the postulated fragmentation pathway.

rearrangement leading to the elimination of AOZ and the formation of the ion at *m*/*z* 134. These assignments were consistent with the peaks observed in the product ion mode spectrum of [$^{13}C_6$]NBAOZ with *m*/*z* 242 as the precursor ion. Indeed, the fragment ions at *m*/*z* 140, 155, and 198 were observed, and their 6 Da mass excess (with respect to NBAOZ) demonstrated unambiguously that the six carbon atoms of the aromatic ring moiety were still attributed to the structure of these ions. It should be added that such a migration of a nitro group-belonging oxygen atom was already reported in electron impact-mediated fragmentation of aromatic nitro compounds (*25*, *26*). In these cases, the migration of the oxygen atom led to the loss of CO, and consequently a 28 Da mass transition was observed in the mass spectra.

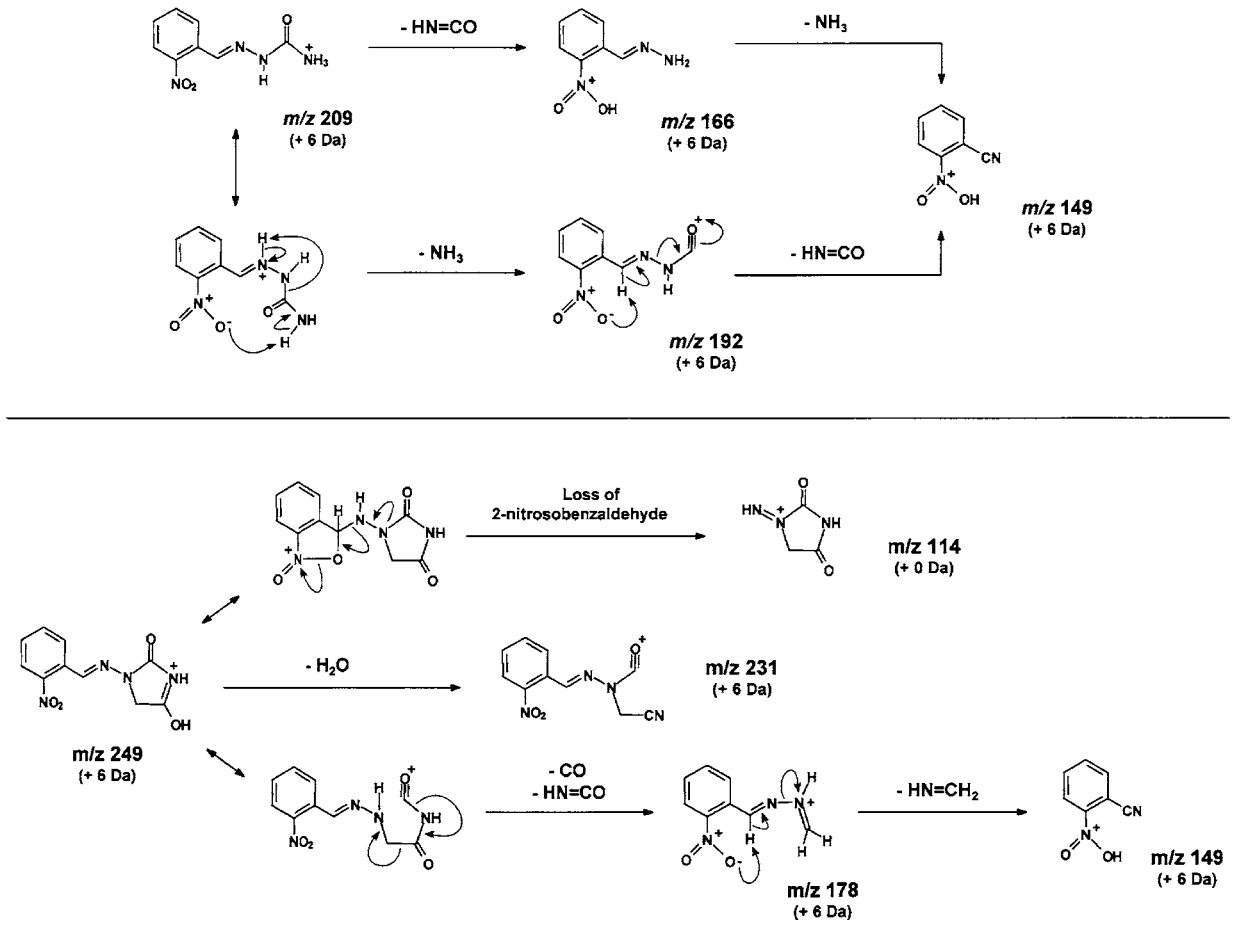

Figure 3. Postulated fragmentation pathway of protonated molecules of either NBSC (top) or NBAH (bottom) (up-mass shift observed with carbon-13 labeled derivatives are indicated in brackets).

The 15 eV product ion mode spectrum of NBAOZ also exhibited two fragments at *m*/*z* 104 and 101. In parallel, the [$^{13}C_6$]NBAOZ spectrum showed the occurrence of fragment ions at *m*/*z* 110 and 101, indicating that the nitrophenyl moiety is eliminated in the process, leading to the formation of the ion at *m*/*z* 101, and not in the case of the ion at *m*/*z* 104. The ion at *m*/*z* 101 was observed only on the MS^2 spectrum recorded with the ion trap mass spectrometer (*m*/*z* 236 as the precursor ion), so it suggested that it arose from the degradation of the five-membered ring transient species with a concomitant elimination of 2-nitrosobenzaldehyde. The ion at *m*/*z* 104 was observed when the ion trap analyzer-based spectrum was recorded in the MS^3 mode with the acquisition set at *m*/*z* 236 → *m*/*z* 134 → *m*/*z* 50−250. Therefore, the ion at *m*/*z* 104 could originate from the elimination of the radical ON•, giving rise to a radical cation containing the six carbon atoms of the nitrophenyl moiety. The loss of the radical ON• was reported previously in the case of the fragmentation of nitrobenzene derivatives (*27*−*29*).

NBSC and NBAH. Similarly NBSC, [$^{13}C_6$]NBSC, NBAH, and [$^{13}C_6$]NBAH were used for the assignment of the fragment ions and the elucidation of the fragmentation pathway. The spectra of both NBSC and NBAH exhibited fragment ions at *m*/*z* 134 and 104, the formation of which can be explained by the same process as the one postulated for NBAOZ. The fragment ion at *m*/*z* 149 was also assigned to be the protonated 2-cyanonitrobenzene.

The product ion mode spectrum of NBAH (*m*/*z* 249 as the precursor ion) exhibited two ions at *m*/*z* 231 and 178 (both present in the MS^2 ion trap-recorded spectrum), with a 6 Da up-mass shift when [$^{13}C_6$]NBAH was used (**Figure 3**). The ion at *m*/*z* 231 was suggested to be due to a water elimination from the metabolite moiety. The formation of the ion at *m*/*z* 178 can be explained in terms of a concomitant loss of CO and HN═CO from the metabolite moiety. When the CID spectrum of [$^{13}C_6$]NBAH was recorded, an ion at *m*/*z* 114 was observed with no up-mass shift compared to the unlabeled NBA derivative of 3-aminohydantoin. This clearly demonstrated that the NBA moiety was eliminated during the process, and the ion at *m*/*z* 114 was assigned to be the protonated 1-aminohydantoin.

In the case of NBSC, the CID spectrum recorded on the ion trap mass spectrometer with the precursor ion set at *m*/*z* 209 ($[M + H]^+$) exhibited fragment ions at *m*/*z* 192, 166, and 134. The occurrence of an ion at *m*/*z* 192 was postulated to be due to the loss of NH_3, whereas the elimination of HN═CO afforded the fragment ion at *m*/*z* 166. This assignment was consistent with the comparison of the spectra obtained with NBSC and [$^{13}C_6$]NBSC on the triple-stage mass spectrometer. Interestingly, the fragment ion at *m*/*z* 149 was observed in MS^3 spectra when the ion trap mass spectrometer was set on either *m*/*z* 209 → *m*/*z* 192 → *m*/*z* 50−230 or *m*/*z* 209 → *m*/*z* 166 → *m*/*z* 50−230 acquisition mode. This clearly indicated that the ion at *m*/*z* 149 could be formed by either an elimination of HN═CO from ion at *m*/*z* 192 or a loss of NH_3 from the ion at *m*/*z* 166.

NBAMOZ. In the case of NBAMOZ, no fragment ions at *m*/*z* 149, 134, and 104 were observed in the CID spectrum, but two intense ions at *m*/*z* 291 and 262 were present when the collision energy was set at 15 eV (**Figure 4**). These ions were at *m*/*z* 297 and 268 when the spectrum was recorded with [$^{13}C_6$]-NBAMOZ (indicating losses of atoms belonging to the nitrofuran metabolite moiety), and the spectra obtained on the ion

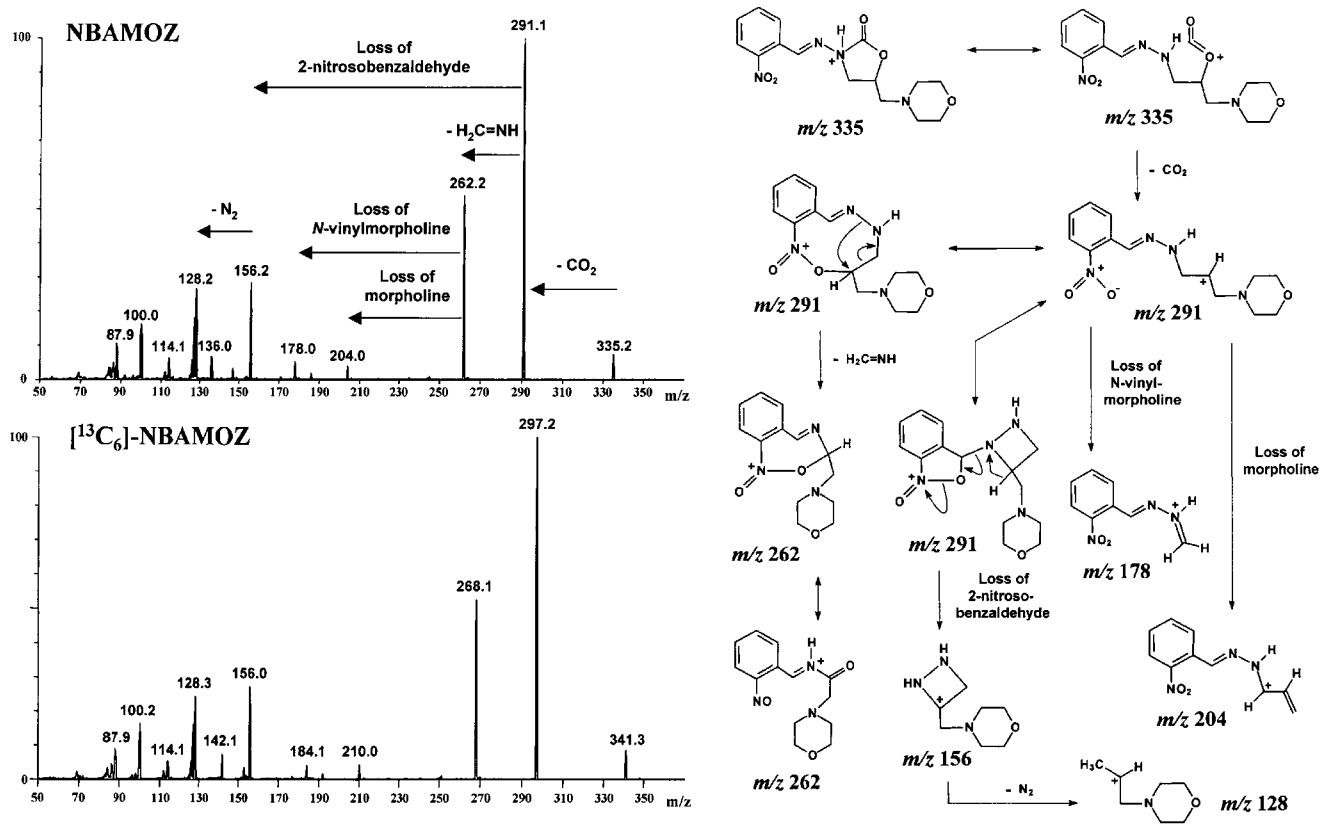

Figure 4. CID spectra of both NBAMOZ and [$^{13}C_6$]NBAMOZ, recorded at 15 eV, and the postulated fragmentation pathway.

trap mass spectrometer demonstrated unambiguously that the ion at *m*/*z* 291 was generated by the degradation of the protonated molecule, whereas the ion at *m*/*z* 262 arose from the fragmentation of the ion at *m*/*z* 291. From these results it was postulated that the loss of CO_2 from the oxazolidinone ring generated the carbocation with the charge located on the atom bond to the methylene bridge between the oxazolidinone and morpholino rings (*m*/*z* 291). This carbocation could subsequently rearrange into a transient species with a nine-membered ring involving the nitro group. This species would undergo a fast degradation process leading to the loss of $H_2C{=}NH$, generating the fragment ion at *m*/*z* 262.

Fragment ions at *m*/*z* 204 and 178 were also observed in the CID spectrum of NBAMOZ and, according to the data obtained with [$^{13}C_6$]NBAMOZ (both ions with a 6 Da up-mass shift), their formations were proposed to be due to the respective eliminations of morpholine and *N*-vinylmorpholine from the carbocation at *m*/*z* 291. This fragmentation pathway was consistent with the occurrence of ions at *m*/*z* 204 and 178 in the ion trap mass spectrometer-based MS^3 spectrum with acquisition conditions set at *m*/*z* 335 → *m*/*z* 291 → *m*/*z* 50−350. In contrast, no up-mass shift was observed for the ions at *m*/*z* 156 and 128 when spectra were recorded with [$^{13}C_6$]-NBAMOZ instead of NBAMOZ. The formation of a four-membered ring involving two carbon and two nitrogen atoms together with the generation of a covalent bond between the carbon atom in the α-position of the aromatic ring and an oxygen atom of the nitro group (carbocation at *m*/*z* 291) could explain the occurrence of the fragment ion at *m*/*z* 156. Indeed, the migration of a hydrogen atom belonging to the four-membered ring of the imino nitrogen could promote an elimination of 2-nitrosobenzaldehyde, giving rise to the carbocation at *m*/*z* 156. A subsequent loss of dinitrogen from this species could explain the formation of the ion at *m*/*z* 128. This pathway was consistent with the findings obtained with the ion trap mass spectrometer; the ion at *m*/*z* 156 was shown to be generated by the fragmentation of the ion at *m*/*z* 291, and the ion at *m*/*z* 128 was a product ion of the fragment at *m*/*z* 156.

NMR Analysis. The NMR spectra of the NBA derivatives of nitrofuran metabolites showed several common features due to the presence of the nitrophenyl ring moiety and the iminyl group. The chemical shifts and coupling constants of the nitrophenyl ring and the iminyl group resonance signals of the four different metabolites were very similar. The complete assignments of the proton and carbon signals of NBAOZ, NBSC, NBAH, and NBAMOZ were achieved using the data of the ^1H, ^{13}C, and DEPT 135 spectra, together with the COSY, COSY long-range, and heteronuclear two-dimensional techniques (HSQC and HMBC).

Nitrophenyl Ring and Iminyl Moiety. The ^{13}C NMR spectra of either NBAOZ, NBSC, NBAH, or NBAMOZ exhibited six resonance signals corresponding to the carbon atoms of the aromatic ring (**Table 1**). The DEPT 135 spectra provided clear evidence that the two signals at approximately δ 128 and 148 were due to quaternary carbons. Because it is very well-known that the nitro group generates a downfield shift of about $\Delta\delta = +20$ (*30*), the signal resonating at δ 148 was assigned to the carbon atom C-2. As a consequence, the signal at δ 128, was assigned to the carbon C-1. The four other signals were assigned with the values given by the simulation (accuracy ranging from −1.7 to 0.55%). The assignment of the four signals observed in the ^1H NMR spectra of either NBAOZ, NBSC, NBAH, or NBAMOZ (**Table 2**) was inferred from the HSQC analysis. Further support for this assignment was obtained on the basis of the coupling constants and the correlation peaks in the COSY map. The coupling constant values (**Table 3**) within the range of 7−9 Hz were strongly indicative of a vicinal coupling, whereas the values at 1−3 Hz were typical of a ^4J coupling

Table 1. Carbon-13 Chemical Shifts (δ Referenced to TMS) of NBAOZ, NBSC, NBAH, and NBAMOZ in DMSO-d_6 at Room Temperature

	NBAOZ	NBSC	NBAH	NBAMOZ
nitrobenzyl moiety				
1	128.4	128.7	128.3	128.4
2	148.0	147.6	148.0	148.1
3	124.7	124.3	124.7	124.7
4	130.4	129.4	130.4	130.5
5	133.7	133.1	133.7	133.7
6	128.2	127.8	128.0	128.1
–CH═N	139.0	133.8	138.1	139.1
metabolite moiety				
C═O	153.6	156.3	153.3	153.0
N—CH_2–	42.0		48.8	45.6
O—CH_2–, O—CH, or C═O	61.7		168.6	71.2
–CH_2– (mmg)[a]				60.7
N—CH_2– (mmg)				53.7
O—CH_2– (mmg)				66.0

[a] mmg = morpholinomethyl group.

Table 2. Proton Chemical Shifts (δ Referenced to TMS) of NBAOZ, NBSC, NBAH, and NBAMOZ in DMSO-d_6 at 21 °C

	NBAOZ	NBSC	NBAH	NBAMOZ
nitrobenzyl moiety				
3	8.08, dd[a]	7.99, dd	8.07, dd	8.08, dd
4	7.68, ddd	7.58, ddd	7.67, ddd	7.68, ddd
5	7.82, ddd	7.72, m	7.82, ddd	7.82, m
6	8.02, dd	8.34, dd	8.02, dd	8.02, dd
–CH═N	8.09, s	8.23, s	8.05, s	8.09, s
metabolite moiety				
N—CH_2–	3.95, m		4.36, s	3.65, dd; 4.06, dd
O—CH_2– or O—CH	4.52, m			4.94, m
–NH–		10.65, s	11.38, s	
–NH_2		6.62, s		
–CH_2– (mmg)[b]				2.69, d
N—CH_2– (mmg)				2.49
O—CH_2– (mmg)				3.58, t

[a] Multiplicity abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet. [b] mmg = morpholinomethyl group

Table 3. ^3J(H,H) and ^4J(H,H) Coupling Constants (in Hertz) of the Aromatic Ring of NBAOZ, NBSC, NBAH, and NBAMOZ in DMSO-d_6 at 21 °C

atom labeling	NBAOZ	NBSC	NBAH	NBAMOZ
3–4	8.1	8.1	8.1	8.2
4–5	7.2	7.5	7.5	7.4
5–6	7.9	7.9	7.9	7.9
3–5	1.1	1.1	1.1	1.3
4–6	1.3	1.3	1.5	1.5

(*31*). The iminyl group gave rise to a signal at δ 8.0−8.3 in the ^{1}H NMR spectrum and at δ 133−140 in the ^{13}C NMR spectrum. These typical signals were used to assign some atoms characteristic of the metabolite moiety of the derivatives.

NBAOZ Metabolite Moiety. The ^{1}H NMR spectrum of NBAOZ exhibited two multiplets at δ 3.95 (N−CH_2) and δ 4.52 (O−CH_2), corresponding to the two methylene groups of the oxazolidinone ring. The unambiguous assignment of the signals was accomplished with a COSY long-range experiment, which exhibited a cross-peak between the signal of the iminyl proton (δ 8.09) and the signal resonating at δ 3.95. This result was confirmed by the HSQC experiment, which provided a map showing a cross-peak between the signal at δ 3.95 and the one at δ 42.0 corresponding to the carbon N−CH_2− (simulation: δ 42.3) and a second one between the signal at δ 4.52 and the carbon O−CH_2− resonating at δ 61.7 (simulation: δ 61.8). The quaternary carbon of the carbonyl group of the oxazolidinone ring showed a signal at δ 153.6 in the ^{13}C NMR spectrum.

NBSC Metabolite Moiety. Two broad singlets observed at δ 6.62 and 10.65 in the ^{1}H NMR spectrum were assigned to −NH_2 and −NH−, respectively. This assignment was consistent with the occurrence of a cross-peak observed in the COSY map between the signal at δ 10.65 and the one at δ 8.23 (hydrogen of the iminyl group). The ^{13}C spectrum exhibited a single resonance signal at δ 156.3 corresponding to the carbonyl group of the semicarbazide moiety of NBSC.

NBAH Metabolite Moiety. According to the integral ratios, the ^{1}H NMR spectrum showed the occurrence of three protons. The broad singlet at δ 11.38 was assigned to the hydrogen −NH− and the signal at δ 4.36 to the methylene group of the hydantoin ring. This assignment was supported by the observation of a correlation peak in the COSY long-range map between the hydrogen atoms at δ 4.36 (CH_2) and the one of the iminyl group (δ 8.05). In the HSQC map, the signal at δ 4.36 correlated with the carbon atom resonating at δ 48.8. The two carbonyl groups provided two signals at δ 153.3 (N−CO−NH) and 168.6 (CH_2−CO−NH) in the ^{13}C spectrum of NBAH whose assignments were achieved with the data of the HMBC experiment. This was consistent with the data of the simulation that provided theoretical chemical shifts at 156.5 ppm for N−CO−NH and at 168.6 ppm for CH_2−CO−NH.

NBAMOZ Metabolite Moiety. The integration of the signals in the ^{1}H NMR spectrum led to the following relative intensities 1:1:1:4:2, corresponding to nine hydrogen atoms for the metabolite moiety. The four additional hydrogen atoms belonging to methylene groups of the morpholino ring could not be observed in the ^{1}H NMR spectrum because their signals were hidden by a signal of the solvent at δ 2.50. The COSY (**Figure 5**) exhibited correlation peaks between the signal of the iminyl moiety (δ 8.09) and each of the signals resonating at δ 3.65 and 4.06, which was rationalized in terms of the occurrence of a scalar coupling of the iminyl hydrogen with the latter methylenic hydrogens of the oxazolidinone ring. This was further supported by the presence of a highly intense cross-peak between the two hydrogens at δ 3.65 and 4.06 and the strong coupling constant (J = 8.9 Hz) measured between these two signals, which were indicative of a *geminal* coupling. The correlation map also exhibited two cross-peaks between the signals at δ 3.65 and 4.06 on the one hand and the signal at δ 4.94 on the other. This observation demonstrated unambiguously that the signal at δ 4.94 was accounted for by the hydrogen CH_2−CH−O. This assignment was supported by the data of the HSQC experiment together with the DEPT 135 spectrum. Indeed, the HSQC map exhibited a cross-peak between the hydrogen resonating at δ 4.94 and the carbon at δ 71.2 (showing a positive response in the DEPT 135 spectrum). The hydrogen atoms of the methylene bridge between the oxazolidinone and the morpholino rings exhibited a doublet centered at δ 2.69 (correlated with the carbon at δ 60.7 in the HSQC map) in the ^{1}H NMR spectrum and a correlation peak with the signal resonating at δ 4.94 in the map of the COSY experiment. In the case of the morpholino ring, only the hydrogen atoms O−CH_2− could be observed at δ 3.58 in the one-dimensional ^{1}H NMR spectrum because the signal of the protons N−CH_2− were interfering with a signal due to the solvent (δ ~2.5). However, the correlation map of the COSY experiment clearly showed a cross-peak between the signal at δ 3.58 and the hidden one resonating at δ 2.49. This observation allowed us to assign the latter signal to the hydrogens N−CH_2−. The HSQC map

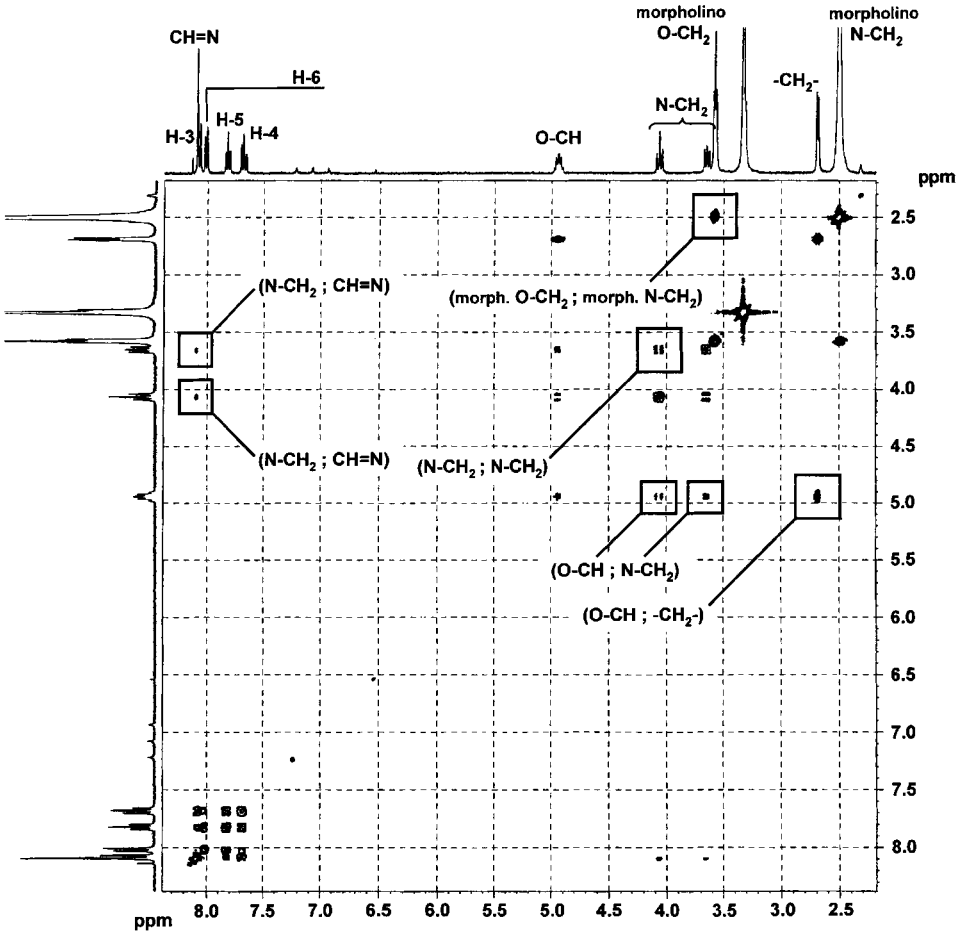

Figure 5. Two-dimensional homonuclear shift correlation with gradient selection (COSY) map of NBAMOZ in DMSO-d_6 (21 °C).

showed that the signal at δ 3.58 was correlating with the carbon O−CH_2− resonating at δ 66.0 (simulation: δ 66.6) and the one at δ 2.49 with the carbon N−CH_2− resonating at δ 53.7 (simulation: δ 54.0).

Conformational Analysis. The conformational study of NBAOZ, NBSC, NBAH, and NBAMOZ in DMSO was carried out on the basis of the homonuclear NOE difference spectroscopy (*32*). For NBAOZ, NBAH, and NBAMOZ the NOE difference spectra exhibited a spatial correlation between the iminyl hydrogen (δ 8.05 or 8.09) and the hydrogen atoms N−CH_2− (δ 3.95, 4.36 or 3.65, 4.06, respectively). In the case of NBSC, a similar correlation was observed between the iminyl hydrogen (δ 8.23) and the hydrogen atom N−H (δ 10.65). These findings are strongly indicative of an *E* configuration (*33*).

Conclusion. We describe a convenient synthetic route to procure the four carbon-13-labeled NBA derivatives of AOZ, AH, SC, and AMOZ. Our approach encompasses the preparation of [$^{13}C_6$]NBA prior to derivatization with the metabolites of nitrofuran antibiotics, and a major advantage is that the label can potentially be extended to the preparation of the derivatives of other metabolites of the nitrofuran family of antibiotics (*34*). Furthermore, the derivatives are fully characterized by performing detailed fragmentation studies by tandem MS. Such stable isotope-labeled compounds are suitable in the development of LC-MS-based methods to match the validation criteria of analytical methods dedicated to the determination of trace levels of antibiotic residues in foods (*35*). In particular, employment of isotope dilution techniques in LC-MS analysis compensates for analyte losses during extraction and cleanup, as well as for matrix-induced suppression of ionization (*16*).

Further work on the analytical methodology for the detection of trace levels of metabolites of nitrofuran antibiotics in food of animal origin based on isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry is being conducted in our laboratory and will be communicated in due course.

SAFETY

Uncontrolled nitration of toluene may lead to the formation of trinitrotoluene, a very explosive compound. Extreme attention should be paid to the molar ratio of toluene to nitronium tetrafluoroborate in the reaction mixture. Chromium trioxide is a strong corrosive oxidizing agent, which can cause cancer by inhalation and damage the environment.

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