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Note

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Aristoxazole Analogues. Conversion of 8-Nitro-1-naphthoic Acid to 2-Methylnaphtho[1,2-*d*]oxazole-9-carboxylic Acid: Comments on the Chemical Mechanism of Formation of DNA Adducts by the Aristolochic Acids

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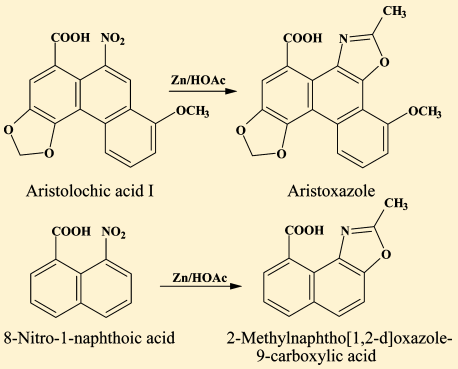
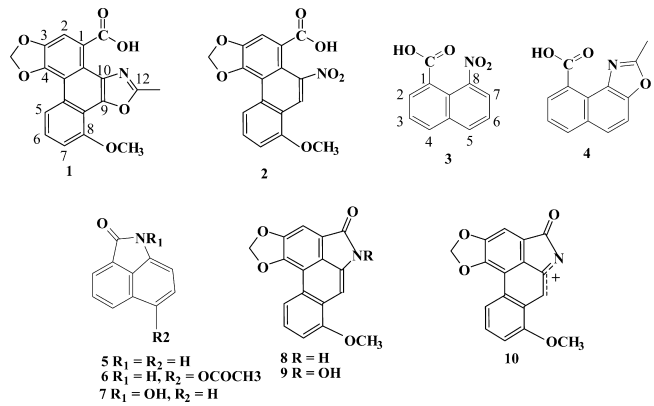
Supporting Information

ABSTRACT: 2-Methylnaphtho[1,2-*d*]oxazole-9-carboxylic acid was obtained by reduction of 8-nitro-1-naphthoic acid with zinc−acetic acid. This naphthoxazole is a condensation product between an 8-nitro-1-naphthoic acid reduction intermediate and acetic acid and is a lower homologue of aristoxazole, a similar condensation product of aristolochic acid I with acetic acid that was previously reported. Both oxazoles are believed to arise via a common nitrenium/carbocation ion mechanism that is likely related to that which leads to aristolochic acid−DNA−adducts.



Aristoxazole (**1**) is a phenanthroxazole that arises from a condensation of aristolochic acid I (**2**) (AA-I; 8-methoxy-6-nitrophenanthro[3,4-*d*][1,3]dioxole-5-carboxylic acid) with acetic acid and is formed when AA-I (**2**) is reduced with Zn in hot acetic acid.[1] In this Note we report that the same reaction can be demonstrated with the simpler 8-nitro-1-naphthoic acid (NNA; **3**) under the same reaction conditions, and this leads to the corresponding naphthoxazole **4** (2-methylnaphtho[1,2-*d*]oxazole-9-carboxylic acid). The formation of this compound shows that aristolochic acid I (**2**) and NNA (**3**) display the same behavior when reduced with Zn/acetic acid. Consequently, the only requirement for the reaction to proceed seems to be the presence of the carboxylic and nitro groups located in a *peri* relationship. The present study indicates that this reaction can be extended to other polycyclic aromatic hydrocarbons bearing a nitro and a carboxyl group in the same positional relationship. The present finding is also important in relation to DNA−adduct formation by the AAs, because AA−adducts and the oxazoles seem to be generated by the same mechanistic pathway (see discussion below).

It is known that reduction of NNA (**3**) produces the lactam **5** {benz[*c*,*d*]indol-2(1*H*)-one} by spontaneous cyclization of the reduction product 8-aminonaphthalene-1-carboxylic acid (reduction is instantly achieved by Fe^{2+} ions in aqueous solution at room temperature).[2,3] In this study, reduction of NNA with Zn/HOAc at room temperature also yielded the expected lactam **5**. However, when the reaction is carried out in boiling acetic acid, in addition to lactam **5**, two new reduction products



are formed, namely, the naphthoxazole **4** and the acetoxy derivative **6**. The major reaction product is the lactam **5** (ca. 46%), which is accompanied by minor amounts of the 5-acetoxy derivative **6** (ca. 27%) and the oxazole **4** (ca. 12%), as determined by HPLC analysis. Unidentified products (ca. 15%) were also formed.

Compound **4** was isolated from the reaction mixture by extraction with aqueous $NaHCO_3$ solution, whereas compounds **5** and **6** were separated by preparative TLC. Compound **6**, $C_{13}H_9NO_3$, was found to be 2-oxo-1,2-

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dihydrobenzo[*c,d*]indol-6-yl acetate. The possible formation of the corresponding isomer with the acetoxy group at position 7 of the lactam **5** can be eliminated on the basis of the NMR data (Table 1).

Table 1. ^{1}H NMR and ^{13}C NMR Data for 2-Methylnaphtho[1,2-*d*]oxazole-9-carboxylic Acid (4), Benz[*c,d*]indol-2(1*H*)-one (5), and 2-Oxo-1,2-dihydrobenzo[*c,d*]indol-6-yl Acetate (6) in DMSO-d_6 (δ in parts per million, *J* in parentheses in herz)

	4		5		6	
position	δH	δC[a]	δH	δC[a]	δH	δC[a]
1		130.4		127.1		127.1
2	7.69 dd (7.2, 1.2)	125.8	8.01 d (6.8)	123.6	8.08 d (6.8)	126.2
3	7.58 dd (8.4, 7.2)	125.5	7.77 dd (8.0, 6.8)	128.8	7.85 dd (8.2, 6.8)	129.3
4	8.16 dd (8.4, 1.2)	130.1	8.13 d (8.0)	130.8	8.10 d (8.2)	124.3
4a		130.8		128.2		123.4
5	7.97 d (8.8)	124.4	7.56 d (8.4)	119.4		141.2
6	7.93 d (8.8)	111.6	7.48 dd (8.4, 6.8)	129.0	7.24 d (7.6)	120.8
7		148.3	6.99 d (6.8)	106.1	6.98 d (7.6)	105.9
8		134.8		138.1		136.0
8a		121.1		125.6		126.1
C=N		162.3				
1-COOH	13.25 s (broad)	170.4				
CH_3	2.68 s	14.2				
8-NH			10.80 s		10.86 s	
1-C=O				169.0		169.8
$CH_3\underline{C}O$						168.8
$\underline{CH_3}CO$						20.6

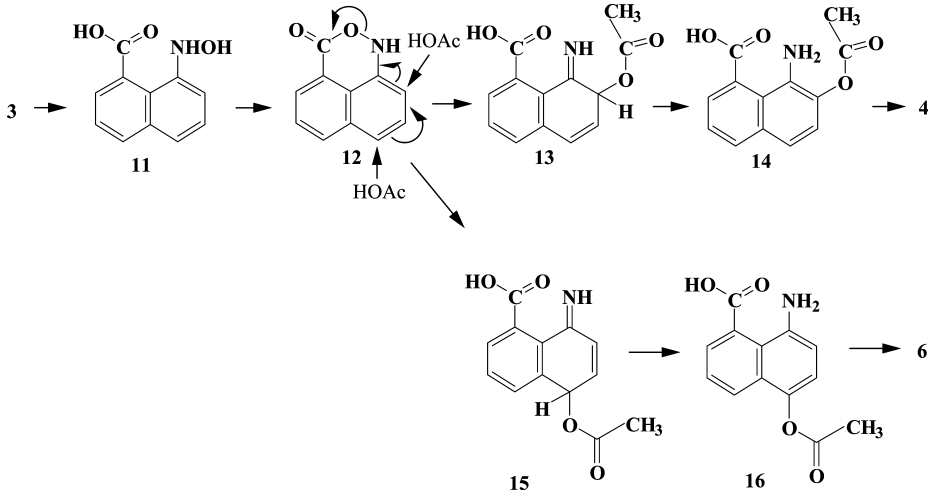
[a]Assigments of signals with similar chemical shifts may be reversed.

Compound **4**, $C_{13}H_9NO_3$, is a carboxylic acid that was obtained as a white, crystalline solid. ESIMS displayed a protonated molecular ion at *m/z* 228. The ^{1}H and ^{13}C NMR data of this compound are shown in Table 1. The oxazole structure in this molecule was corroborated by carbon signals at δ_C 134.8, 148.3, and 162.3, which are coherent with those of other oxazole derivatives.[1] The position of CH_3-10 at δ_C 14.2 matches that of the corresponding methyl group in 2-methylbenzoxazole (δ_C 14.4), thus eliminating the possibility that the methyl is part of an an acetyl group. The above considerations together with evidence gathered from other oxazole derivatives[1] support structure **4** for this reduction product of NNA.

The biological activity of NNA (**3**) has been previously studied by Pfau et al.[4] NNA (**3**) displays a mutagenic activity similar to AA-I (**2**) in *Salmonella typhimurium* strain TA 100 in the Ames assay.[4] Since compounds such as 3-nitro-2-naphthoic acid are devoid of mutagenic activity, Pfau et al. concluded that the mutagenicity is associated with compounds in which the carboxyl and nitro groups are *peri* positioned as in **2** and **3**. Molecular orbital calculations of AA-I (**2**) and NNA (**3**) showed that the nitro and carboxyl groups are in a similar compressed situation (the aromatic version of $A^{1,3}$-strain)[5] in these molecules.[4] Pfau et al. proposed that the observed mutagenic activity of *peri*-substituted nitro aromatic carboxylic acids (**2**, **3**) is due to the reductive formation of a cyclic hydroxamic acid (= *N*-hydroxylactam; **7**, **9**), which by solvolysis generates a cyclic nitrenium ion (e.g., **10**) with delocalized positive charge. The nitrenium ion reacts, through the carbon in *ortho* position to the N-substitution, with nucleophiles such as the exocyclic amino groups of purine nucleotides in DNA to form adducts.[4,6]

On the basis of the mechanism of metabolic activation of *peri*-substituted nitro aromatic carboxylic acids proposed by Pfau et al.,[4] the formation of naphthoxazole **4** could be explained as arising from the **3** → **7** → nitrenium ion → **4** pathway. However, generation of **4** via this mechanism appears to be quite unlikely in view of the considerations discussed in a previous paper.[1] An alternative mechanism for the formation of the oxazole **4** is proposed in Scheme 1. It may be assumed that the NOH group of the *N*-hydroxy intermediate **11** can react with the COOH either to give the lactam **7** or through the OH group to yield the dihydronaphthoxazinone **12**. Compound **7** would be further reduced to the lactam **5**. On the other hand, the dihydronaphthoxazinone **12** can either be reduced to give **5** or undergo a solvolysis to a nitrenium/carbocation ion, which then acquires a nucleophile such as acetate to generate the

Scheme 1. Proposed Mechanism for the Formation of 2-Methylnaphtho[1,2-*d*]oxazole-9-carboxylic Acid (4) and 2-Oxo-1,2-dihydrobenzo[*c,d*]indol-6-yl Acetate (6) from 8-Nitro-1-naphthoic Acid (3)



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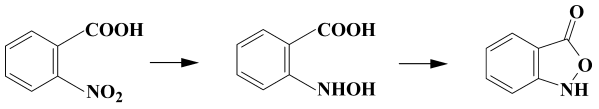
addition compounds at C-5 or C-7 of the naphthalene nucleus. This is in contrast to the AAs, in which only C-9, *ortho* to the nitrogen, is involved. HPLC profiles and amounts of the purified reduction products **4** and **6** recovered from the reaction mixture indicate that the initial adduct **15** was formed in higher proportion than the isomer **13**. After formation of the initial adduct **13**, aromatization to **14** and condensation would then produce the naphthoxazole **4**. The 5-acetoxy intermediate **16** is converted and isolated as the lactam **6**. Among the reaction products, the assumed 7-acetoxy derivative of the lactam **5** could not be isolated. This suggests that the **13** → **14** → **4** pathway may proceed rapidly; that is, addition, aromatization, and condensation may occur in quick succession, so that lactamization by reaction of the 8-amino group with the 1-carboxyl group does not take place. This may especially be true if the carboxyl group is present in the unreactive anionic form of the zinc salt. Thus, a significant reduction product of NNA (**3**) is the oxazole **4**. There is no evidence for the formation of the assumed intermediate **12**. However, compounds such as *o*-nitrobenzoic acid (**17**), under reduction conditions, can give the benzo[*c*]isoxazolin-3-one (**19**) by cyclization of the reduction intermediate 2-(hydroxylamino)-benzoic acid (**18**) (Scheme 2).[7] This reaction supports the same type of cyclization for the formation of **12** from **11**.

Scheme 2. Synthesis of Benzo[*c*]isoxazolin-3-one (19) from *o*-Nitrobenzoic Acid (17)[7]



17 18 19

This study shows that the carboxyl group may be essential for the formation of the naphthoxazole **4** and by extension of the phenanthroxazoles and AA-DNA adducts; otherwise the simple 1-nitronaphthalene, or nitro aromatic compounds in general, could give the oxazole structure by reduction with Zn/HOAc, which is not the case. In the present Note it is suggested that the COOH participates in the activation of the molecule by forming a dihydro-oxazinone structure such as **12**. The 1-COOH group may also interact with the *peri*-nitrogenated function at C-8 in other ways, e.g., by forming a lactam structure as represented in **5** and **7**. As stated above, in order to rationalize the observed mutagenic activity of *peri*-substituted nitro-carboxylic acids and DNA adduct formation, Pfau et al. suggested[4] that ring closure of the uncyclized precursors, such as the *N*-hydroxylamine **11**, to give compounds **7** and **9**, occurs because of steric stress. This also likely contributes to stabilization of the nitrenium ion, the ultimate carcinogen, by delocalization of the positive charge.[4,6] This hypothesis is questionable because (a) solvolysis of the *N*-hydroxylactam **7** would be less likely than **12** to give a nitrenium ion and (b) the conversion of **7** into oxazole **4** is, mechanistically, extremely difficult to explain otherwise. These and other reasons discussed in ref 1 point to the more rational formation of an oxazinone ring intermediate (**12**) for this reaction to proceed. Thus, nitrenium ion formation via dihydrooxazinone (**12**) is likely the mechanistic pathway that leads to **4** and **6** through interaction with HOAc. This also suggests that dihydrooxazinone formation may represent an alternative pathway for the formation of DNA adducts derived from the aristolochic acids (Scheme 3).

Biological Implication. The equal mutagenic potency of NNA (**3**) and AA-I (**2**) in Ames assays with *Salmonella thyphimurium* TA100 shows that both compounds are metabolized in a similar way and to the same extent by this microorganism. The properties of NNA (**3**) and AA-I (**2**) as mutagens are likely due to DNA binding. These results also suggest that they exert their effect via a structurally related reactive intermediate, possibly the corresponding dihydrooxazinones described above. NNA (**3**) and AA-I (**2**) also show similar behavior by reduction with Zn/HOAc to give oxazole derivatives by addition of a molecule of HOAc. Thus, both compounds exhibit the same biological activity and the same chemical reactivity. Mutagenicity and chemical behavior are associated with an aromatic substructure in which a carboxyl group and a nitro group are located in a *peri* position. The above considerations suggest that NNA may also form adducts with DNA in a similar way to the AAs. Assays will be performed to explore the potential of NNA to generate DNA adducts in vitro. One drawback when studying the metabolism of AA-I, in in vivo and in vitro experiments, is the scarcity of this compound. Chemical modifications of the AA-I molecule are also precluded for this reason and the limited positional reactivity of the phenanthrene core. If NNA forms adducts like the AAs, it could be used as a simpler analogue of AA-I that is readily available and could be conveniently modified and labeled. Consequently, NNA could help to elucidate the intricate mechanisms leading to formation of DNA adducts by aristolochic acids in biological systems. Investigations are also under way to explore the mechanisms underlying this reductive conversion of NNA (**3**) into naphthoxazole **4**.

EXPERIMENTAL SECTION

General Experimental Procedures. NNA was synthesized by a literature procedure.[2] Zinc dust was purchased from Mallinckrodt (8681). NMR spectra were recorded on a Bruker 400 MHz FT-NMR spectrometer in DMSO-d_6 with TMS as internal standard. ESI mass spectra were acquired on a Thermo Scientific LCQ Deca XP MAX instrument. HPLC/PDA analyses were performed with a P4000 Thermo-Finnigan chromatograph (Thermo Electron Corporation, San Jose, CA, USA). Column effluent was monitored at 254 nm with a SpectraSystem UV6000LP variable-wavelength PDA detector. Analytical separations were carried out with a C_{18} RP Hypersil GOLD column (RP5, 250 × 4.6 mm, pore size 5 μm, Thermo Electron Corporation). The mobile phase consisted of 0.1% TFA in MeCN (phase A) and 0.1% TFA in H_2O (phase B). The linear gradient program was as follows: 10% to 100% A over 30 min at a flow rate of 1.0 mL/min. GC-MS analyses were carried out in a Hewlett-Packard model 6890 instrument coupled to a Q-Mass 910 quadrupole selective detector at 70 eV. A fused capillary column was used (DB-5MS, 30 m × 0.25 mm i.d.; film thickness 0.25 μm; J & W Scientific); injection port temperature, 230 °C; split ratio 1:20; detector temperature, 330 °C; carrier gas, helium at 1 mL/min; temperature program: 200 to 300 °C linear increase at 8 °C/min. GC/FID analyses were performed on a Trace GC Ultra apparatus (Thermo Electron Corporation) equipped with a flame ionization detector (FID). The output was recorded using a ChromQuest version 4.1 data system. Analyses were performed on a DB-5MS capillary column, and the conditions were as indicated above.

2-Methylnaphtho[1,2-d]oxazole-9-carboxylic Acid (4). 8-Nitro-1-naphthoic acid (**3**) (300 mg) and Zn (900 mg) were refluxed for 1 h in glacial HOAc (21 mL) containing less than 1% water (FisherBiotech, BP1185-500; Alfa Aesar, Johnson Matthey Company, #33252) with magnetic stirring. The reaction mixture was treated with EtOAc (50 mL) and filtered. The insoluble material was washed with EtOAc and H_2O. The EtOAc phase was washed with H_2O and extracted with 5%

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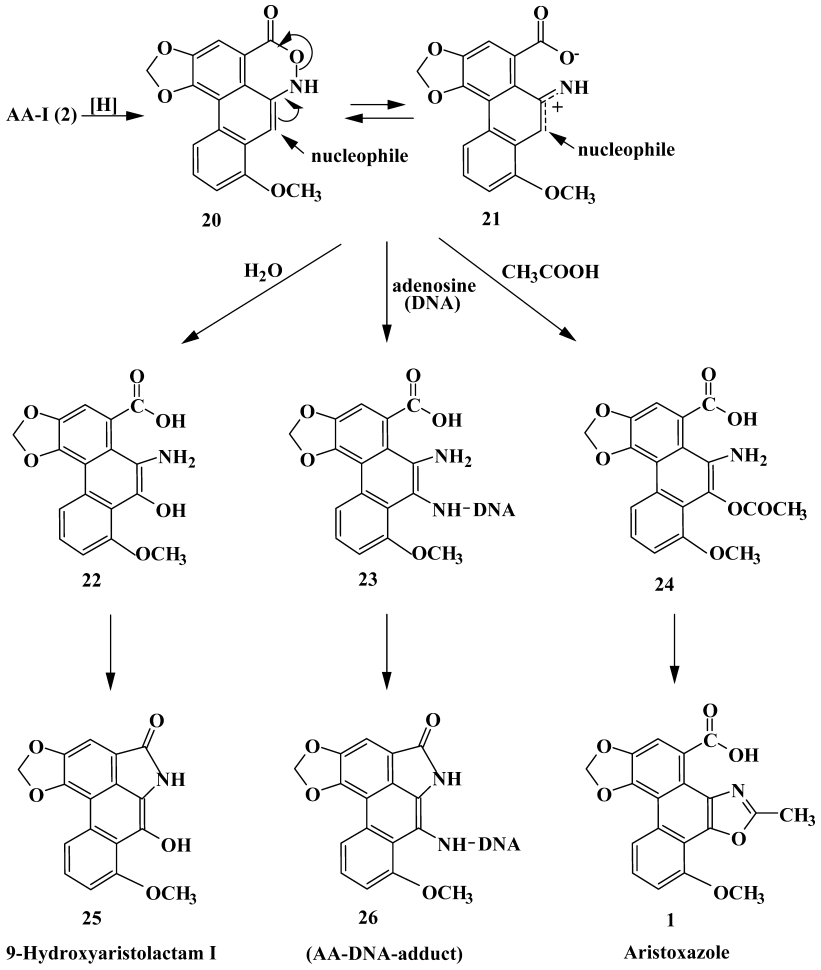
Scheme 3. Proposed Mechanism of Activation and Adduct Formation of Aristolochic Acid I (2)[a]



[a]It was postulated that a dihydrooxazinone (**20**) is the active intermediate formed by reduction of AAs.[1] The dihydrooxazinone (**20**), or its nitrenium ion (**21**), may react with nucleophiles (H_2O, the amino group of adenine and guanine, HOAc) to give the corresponding adducts **22**, **23**, and **24**. These adducts undergo intramolecular cyclization to give the more stable lactam (**25**, **26**) or oxazole (**1**) derivatives. Compound **25** was obtained by in vitro reduction of AA-I with xanthine oxidase.[6] AA-DNA adducts (**26**) are formed by in vitro reduction of AAs with xanthine oxidase in presence of DNA, in rats administered with AAs or in humans exposed to AAs.[6] Compound **1** may be obtained by reduction of AA-I with Zn/HOAc.[1]

aqueous $NaHCO_3$. The $NaHCO_3$ extract was acidified to pH 3 with diluted HCl and extracted with EtOAc. The organic phase was washed with water and evaporated to dryness. In order to remove dark impurities, the residue was dissolved in 20% EtOAc in $CHCl_3$ and the solution passed through a Si gel column (1 g, 70−230 mesh, 60A, Aldrich, catalog no. 28,862-4). The column was washed with 30% EtOAc in $CHCl_3$. Percolate and washes were combined and evaporated to dryness to give a white residue (35.9 mg) of **4**: HPLC, t_R 13.96 min; UV/PAD λ_{max} (nm) 248, 290, 311, 324; ^{1}H NMR and ^{13}C NMR data, see Table 1; ESIMS (MeOH) positive mode, 228.2 $[M + H]^+$, 244.9 $[M + NH_4]^+$; HPLC-ESIHRMS 250.0471 $[M + Na]^+$ (calcd for $C_{13}H_9NO_3Na$, 250.0475), 272.0294 $[M - H + 2Na]^+$ (calcd for $C_{13}H_8NO_3Na_2$, 272.0294).

Separation of Compounds 5 and 6. After separation of the oxazole **4**, a portion of the crude reaction mixture (112 mg) was subjected to preparative TLC (silica gel G, Uniplate, 250 μm, 20 × 20 cm, Analtech, Inc.; 20% EtOAc in $CHCl_3$). After development, two main yellow bands (R_f 0.50 and R_f 0.30) were scraped from the plates and eluted with 20% MeOH in $CHCl_3$. The upper band afforded a yellow residue of the lactam **5** (37 mg), whereas the lower band yielded a yellow residue of the acetoxy derivative **6** (26 mg).

*Benz[c,d]indol-2(1H)-one (**5**):* HPLC, t_R 12.99 min; GC, t_R 6.92 min; UV/PAD λ_{max} (nm) 245, 273, 325 (sh), 337, 359; ^{1}H NMR and ^{13}C NMR data, see Table 1; GC-MS, t_R 17.82 min; *m/z* (rel int) 169 $[M]^+$ (100), 141 $[M - CO]^+$ (23), 140 $[M - CHO]^+$ (35), 114 $[M - CO - CNH]^+$ (23), 113 $[M - CHO - CNH]^+$ (17).

*2-Oxo-1,2-dihydrobenzo[c,d]indol-6-yl Acetate (**6**):* HPLC, t_R 12.66 min; GC, t_R 10.22 min; UV/PAD λ_{max} (nm), 248, 271 (sh), 322, 338, 368; ^{1}H NMR and ^{13}C NMR data, see Table 1; GC-MS, t_R 21.25 min; *m/z* (rel int) 227 $[M]^+$ (7), 185 $[M - H_2C{=}C{=}O]^+$ (100), 156 $[185 - HCO]^+$ (4), 129 $[185 - HCO - CNH]^+$ (17), 101 (11), 75 (8); DART-TOF-MS 228.0651 $[M + H]^+$ (calcd for $C_{13}H_{10}NO_3$, 228.0655), 245.0918 $[M + NH_4]^+$ (calcd for $C_{13}H_{13}N_2O_3$, 245.0921).

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **4** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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