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Synthesis of Partially Deuterated *N*-Nitrosamines – New Standards in Tobacco-smoke Analysis

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Summary. *N*-Nitrosamines of tobacco alkaloids contribute to the cause of tobacco related cancers and, therefore, they are important analytes. Herein the preparation of four partially deuterated *N*-nitrosamines from simple, commercially available, deuterated educts is described, which can serve as useful standards in tobacco-smoke analysis.

Keywords. Isotopic labeling; Heterocycles; Tobacco nitrosamines; Analytical standards; Alkaloids.

Introduction

The effect of tobacco smoke from cigarettes and cigars has been excessively investigated in the last decades. Since then a great number of different illnesses have been directly related to the abuse of tobacco products.

A class of major interest are the tobacco specific *N*-nitrosamines formed during smoking from nicotine and other tobacco alkaloids. Due to their carcinogenic and mutagenic activity, which has been tested positive in different animals, they conceivably contribute to the incidence of tobacco-related cancers [1].

For accurate measurement in tobacco-smoke analysis the use of standards and reference materials is inevitable. Applying partially deuterated analogues is a well established technique and they are highly useful tools in GC/MS and HPLC/MS analysis.

We report herein the synthesis of four partially deuterated *N*-nitrosamines (*NNK*-d_4 (**4**), *NNN*-d_4 (**8**), *NAT*-d_{10} (**11**), *NAB*-d_{10} (**13**)), starting from commercially available deuterated d_4-nicotinic acid ethyl ester and d_5-pyridine.

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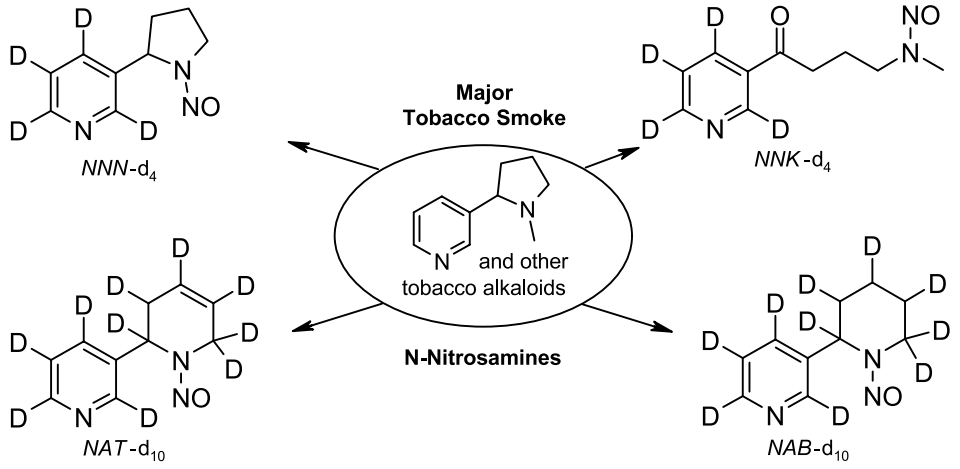


Fig. 1. Major deuterated tobacco-smoke N-nitrosamines

Results and Discussions

*Synthesis of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK-d_4, **4**)*

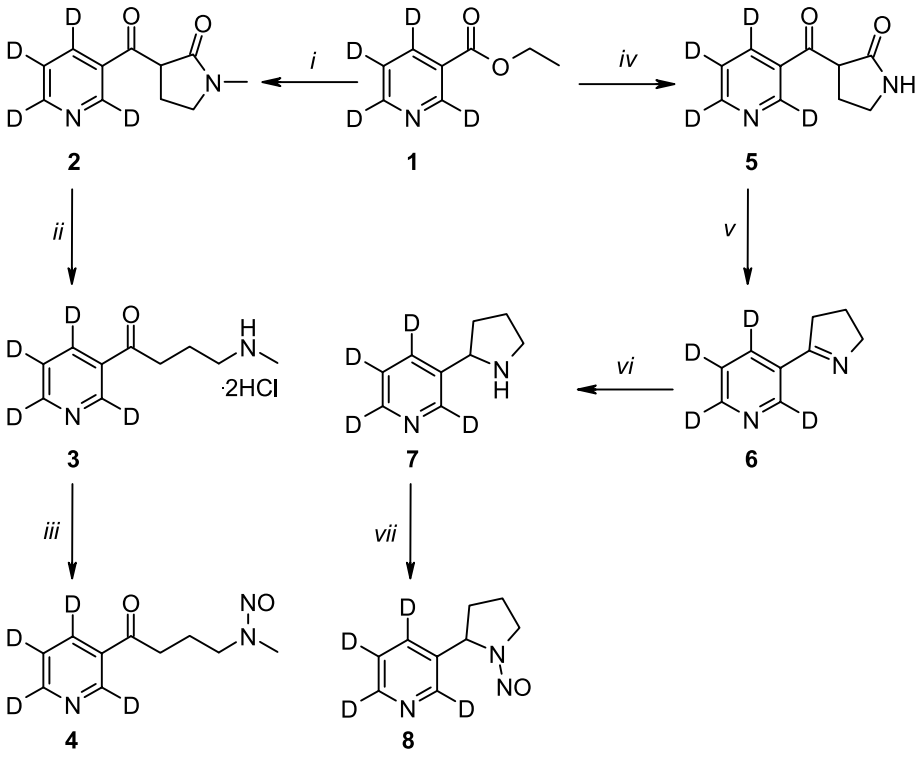
d_4-Nicotinic acid ethyl ester (**1**) was treated with deprotonated *N*-methylpyrrolidin-2-one and the following decarboxylation of the formed β-ketolactam **2** with 6 *N* hydrochloric acid provided the desired amino dihydrochloride **3** [2]. Finally **3** was dissolved in water, the *pH* was adjusted to 4 and after treatment with sodium nitrite the desired d_4-*N*-nitrosamine **4** was obtained. The degree of deuteration was determined *via* HPLC/MS analysis using a protocol which has been already described earlier [3] and proved to be >99%. **4** was obtained in an overall yield of 30% as a mixture of stereoisomers regarding the *N*-nitrosamine moiety (*Z*:*E* = 4.1:1).

*Synthesis of d_4-N-Nitrosonornicotine (NNN-d_4, **8**)*

In case of **8** we also started with **4**, but used the *TMS*-protected pyrrolidin-2-one instead. Decarboxylation of **5** and *in situ* ring closure of the formed intermediate gave myosmine (**6**). Reduction with sodium borohydride afforded nornicotine (**7**) [4], which was finally treated with sodium nitrite/hydrochloric acid [5] to provide the desired *N*-nitrosonornicotine (**8**) in 45% yield and a *Z*:*E* ratio of 1.8:1. The degree of deuteration was determined via HPLC/MS analysis and proved to be >99%.

*Synthesis of d_{10}-N-Nitrosoanatabine (NAT-d_{10}, **11**) and d_{10}-N-Nitrosoanabasine (NAB-d_{10}, **13**)*

In order to obtain almost completely deuterated products we decided to start with d_5-pyridine(**9**)e, introducing the second heterocyclic ring in one step as described



(i) *N*-methyl-2-pyrrolidinone, *LDA*, Et_2O; (ii) 6 *N* HCl, Δ ; (iii) $NaNO_2$, aq. NaOH;
(iv) *N-TMS*-pyrrolidin-2-one, *LDA*, Et_2O; (v) conc. HCl, Δ ; (vi) $NaBH_4$, *Me*OH, *Ac*OH; (vii) $NaNO_2$, 4 *N* HCl;

Scheme 1

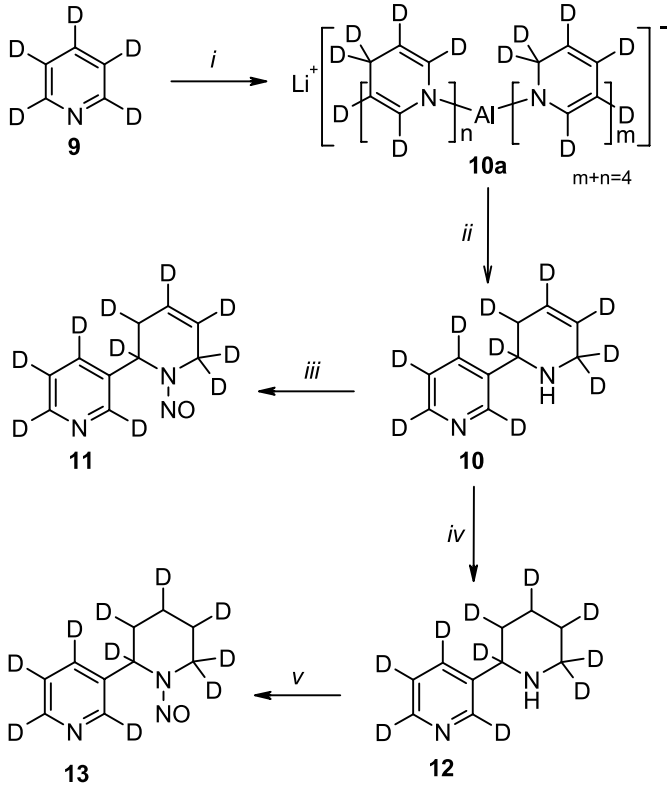
by *Yang* and *Tenner* [6, 7] for nondeuterated anatabine. This was formed by treating pyridine with lithium aluminum hydride. The initially formed *N*-dihydropyridyl complex **10a** is hydrolyzed and subsequently oxidized. Therefore, using d_5-pyridine it was envisaged to obtain the desired d_{10}-anatabine (**10**).

After treatment of lithium aluminum deuteride with an excess of d_5-pyridine, storage for 3 days at $-7°C$ and filtration, complex **10a** was hydrolyzed with H_2O and afterwards oxidized. Although we tried different oxidation procedures, the formation of bipyridine by overoxidation was a major problem in this step. In the end a reaction time of 3 days at room temperature furnished the best results. Furthermore, it was impossible to isolate a pure product at this stage, because bipyridine exhibited a similar chromatographic behavior.

Nevertheless, after final nitrosation pure d_{10}-*N*-nitrosoanatabine (**11**) was obtained after chromatography. Although the product was obtained only in low yield, the procedure proved to be simple giving the target compound in two steps only.

In the case of d_{10}-*N*-nitrosoanabasine (**13**) crude **10** was hydrogenated in the presence of Pd/C. Nitrosation and a final chromatographic purification yielded the desired d_{10}-*N*-nitrosoanabasine (**13**).

For both products a degree of deuteration of >92% was determined.



(i) $LiAlD_4$; (ii) H_2O,O_2; (iii) $NaNO_2$, 4 *N* HCl; (iv) H_2, Pd/C, *Me*OH; (v) $NaNO_2$, 4 *N* HCl

Scheme 2

Experimental

The ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 200 using *TMS* as internal standard. GC Analysis was performed with a CE Instruments TRACE GC using a J & W Scientific-DB-5 column. Liquid chromatography was carried out on a Shimadzu LC-10AD + SPD − 10AV using a Nucleosil 5 C18, 250×2 mm column. Degree of deuteration was determined by HPLC/APCI-MS on an Agilent 1100 series with a C8 Zorbax column. Thin layer chromatography (TLC) was carried out on precoated E. Merck silica gel plates (60F-254) using UV light or molybdato phosphoric acid (5% in ethanol) for visualization. d_5-Pyridine was purchased from Euroiso-top, d_4-nicotinic acid ethyl ester from Cambridge Isotope Laboratories, Inc.

3-(d_4-Nicotinoyl)-1-methyl-pyrrolidin-2-one (**2**, $C_{11}H_8N_2O_2D_4$)

A solution of 2.8 cm^3 of diisopropylamine (20.3 mmol) in 40 cm^3 of anhydrous diethyl ether under Ar was cooled to −78°C and 7.6 cm^3 of *n*-butyl lithium (19 mmol) were added via a syringe. The solution was warmed up to −15°C for 20 min. After cooling again to −78°C 1.6 cm^3 of *N*-methylpyrrolidin-2-one (16 mmol) were added and stirred for 20 min. Compound **1** (2.3 cm^3, 16 mmol) was added dropwise at −78°C. The yellow solution was warmed up to room temperature and stirred overnight. The solution was poured in 70 cm^3 of 2 *N* hydrochloric acid and the aqueous layer was extracted 3 times with ether, in order to remove *N*-methylpyrrolidin-2-one. The aqueous layer was treated with a 10 *N*

NaOH solution to $pH = 6$ and extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered, and the solvent was removed under *vacuo*. The obtained crude product was purified by flash chromatography using CH_2Cl_2:*Me*OH = 50:1 as eluent to yield 3.01 g (89%) of **2** as a brown oil. $R_f = 0.32$ (CH_2Cl_2:*Me*OH = 9:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 4.39$ (dd, $J = 3.7$ Hz, $J = 5.3$ Hz, H-3), 3.65–1.83 (m, 7H, with 2.80 (s, CH_3)) ppm.

4-(Methylamino)-1-(3-(d$_4$-pyridyl)-1-butanone dihydrochloride (**3**, $C_{10}H_{12}N_2OCl_2D_4$)

Compound **2** (2.98 g, 14.3 mmol) was dissolved in 130 cm^3 of 6 *N* HCl and refluxed for 70 h. The solvent was removed *in vacuo* and the brown residue was dissolved in 70 cm^3 of H_2O. The solution was filtered and the solvent was evaporated under reduced pressure. The crude product was crystallized from methanol to yield 2.81 g (78%) of **3** as white crystals. ^{1}H NMR (200 MHz, D_2O): $\delta = 3.27$ (t, $J = 6.75$ Hz, H-2), 3.06 (t, $J = 7.73$ Hz, H-4), 2.66 (s, CH_3), 2.05 (quin, $J = 7.38$ Hz, H-3) ppm.

4-(Methylnitrosamino)-1-(3-(d$_4$-pyridyl)-1-butanone (**4**, $C_{19}H_9N_3O_2D_4$)

Compound **3** (2.73 g, 10.7 mmol) was dissolved in 27 cm^3 of H_2O and cooled to 0°C. A 1 *N* NaOH solution was added to $pH = 4$. A solution of 1.30 g of $NaNO_2$ (18.9 mmol) in 1.6 cm^3 of H_2O was added dropwise at 0°C and the reaction mixture was stirred for 20 h at room temperature. The reaction mixture was extracted with CH_2Cl_2, the combined organic layers were washed three times with 2 *N* NaOH, three times with H_2O, and dried over Na_2SO_4. After filtration the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography using CH_2Cl_2:*Me*OH = 50:1 as eluent to yield 0.98 g (43%) of **4** as light brown crystals. $R_f = 0.42$ (CH_2Cl_2:*Me*OH = 10:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 4.22$ (t, $J = 6.9$ Hz, 1.8H, H-4(trans)), 3.76 (s, 0.3H, CH_3(cis)), 3.71 (t, $J = 7.1$ Hz, 0.2H, H-4(cis)), 3.09–3.04 (m, 4.5H, H-2 und CH_3(trans)), 2.91 (t, $J = 7.1$ Hz, 0.2H, H-2(cis)), 2.22 (quin, 0.2H, H-3(trans)), 1.97 (quin, 1.8H, H-3(cis)) ppm; ^{13}C NMR (50 MHz, $CDCl_3$) $\delta = 197.4$ (C = O), 52.8 (C-4(trans)), 43.8 (C-4(cis)), 38.9 (CH_3(cis)), 35.7 (C-2(cis)), 35.0 (C-2(trans)), 21.7 (C-3(trans)), 19.9 (C-3(cis)); LC/APCI-MS: $R_t = 2.6$ min, $m/z = 212$ $[M]^+$.

3-(d$_4$-Nicotinoyl)pyrrolidin-2-one (**5**, $C_{10}H_6N_2O_2D_4$)

Anhydrous triethylamine (9.1 cm^3, 65 mmol) and 4.5 cm^3 of pyrrolidin-2-one (59 mmol) were added under Ar to 20 cm^3 of anhydrous benzene. After addition of trimethylsilylchloride at room temperature the reaction mixture was stirred for 3 h. It was diluted with 20 cm^3 of anhydrous benzene and filtered under argon. The residue was washed with anhydrous benzene and the filtrate was evaporated at reduced pressure. The crude product was purified by distillation *in vacuo* (93–96°C, 18–22 mbar). A solution of 2.7 cm^3 of diisopropylamine (19 mmol) in 20 cm^3 of anhydrous diethyl ether under Ar was cooled to −78°C and 5.9 cm^3 of *n*-butyl lithium (14 mmol) were added via a syringe. The solution was allowed to warm up to −15°C for 20 min. After cooling again to −78°C 2.44 g of *N-TMS*-pyrrolidin-2-one (15 mmol) were added and it was stirred for 20 min. Compound **1** (1.4 cm^3, 9.7 mmol) was added dropwise at −78°C. The yellow solution was warmed to room temperature and stirred over night. The solution was quenched carefully by addition of 20 cm^3 of H_2O and extracted with 10 cm^3 of diethyl ether. The aqueous layer was treated with 4 *N* HCl to $pH = 7$, saturated with NaCl and extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered, and the solvent was removed under reduced pressure to yield 1.47 g of crude **5**. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 6.46$ (s, NH), 4.24 (m, H-3), 3.34 (m, H-4), 2.23 (m, H-5) ppm.

2,4,5,6-d$_4$-Myosmine (**6**, $C_9H_6N_2D_4$)

Compound **5** (1.47 g, 7.6 mmol) was dissolved in 8.4 cm^3 of 12 *N* HCl (16.8 mmol) and refluxed for 24 h. A 20% NaOH solution was added to adjust $pH = 14$. The reaction mixture was extracted with

CH_2Cl_2 and dried with Na_2SO_4. After filtration the solvent was removed under reduced pressure to yield 0.78 g (68%) of **6** as yellow crystals. $R_f = 0.71$ (CH_2Cl_2:*Me*OH = 5:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 4.2$ (tt, $J = 7.37$, 2.05 Hz, H-3), 3.1 (tt, $J = 8.25$, 2.02 Hz, H-5), 2.2 (m, H-4) ppm.

2,4,5,6-d_4-Nornicotine (**7**, $C_9H_8N_2D_4$)

Myosmine (**6**, 0.78 g, 5.2 mmol) was dissolved in 10 cm^3 of anhydrous methanol and 0.3 cm^3 of acetic acid under N_2 and cooled with a NaCl-ice-bath. After addition of 0.77 g of $NaBH_4$ (20.3 mmol) the reaction was warmed to room temperature and stirred overnight. Since there was still myosmine **6** left, according to TLC, another 0.20 g of $NaBH_4$ (5.2 mmol) were added and the reaction was warmed to 50°C for 2 h. The reaction mixture was quenched by addition of 11 cm^3 of H_2O and extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered, and the solvent was removed under reduced pressure to yield 0.77 g of crude **7**. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 4.9$ (s, NH), 4.1 (m, H-2), 3.3–2.9 (m, H-5), 2.3–1.3 (m, H-3, H-4) ppm.

2,4,5,6-d_4-N-Nitrosonornicotine (**8**, $C_9H_7N_3OD_4$)

Compound **7** (0.77 g, 5 mmol) was dissolved in 11 cm^3 of 10% HCl and cooled to 0°C. A solution of 1.44 g of $NaNO_2$ (2.1 mmol) in 1.9 cm^3 of H_2O was added slowly and the reaction mixture was stirred for 72 h at room temperature. A saturated $NaHCO_3$ solution was added to $pH = 9$. The reaction mixture was extracted with CH_2Cl_2 and dried with Na_2SO_4. After filtration the solvent was evaporated under reduced pressure to obtain 0.79 g (87%) of **8** as a brown oil. $R_f = 0.53$ (CH_2Cl_2:*Me*OH = 5:1); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 5.68$ (t, $J = 6.26$ Hz, 0.64H, H-2′ (cis)), 5.3–5.2 (m, 0.36H, H-2′ (trans)), 4.7–4.6 (m, 0.36H, H-5′ (trans)), 4.5–4.3 (m, 0.36H, H-5′ (trans)), 3.9–3.6 (m, 1.28H, H-5′ (cis)), 2.6–2.3, 2.2–1.9 (2m, 4H, H-3′, H-4′) ppm; LC/APCI-MS: $R_t = 2.6$ min, $m/z = 182$ $[M]^+$.

2,2′,3′,4,4′,5,5′,6,6′,6′-Decadeutero-1,2,3,6-tetrahydro-2,3′-bipyridine (d_{10}-Anatabine) (**10**, $C_{10}H_2N_2D_{10}$)

Lithium aluminum deuteride (4.05 g, 97 mmol) was added under Ar in small portions to 100 cm^3 of stirred d_5-pyridine at −35°C. After stirring for 1 h at room temperature the orange reaction mixture was allowed to rest at −7°C for 72 h. Unreacted lithium aluminum deuteride was filtered off and washed with another 25 cm^3 of d_5-pyridine. The yellow solution was cooled with an ice bath and carefully hydrolyzed with 8 cm^3 of H_2O. After warming to room temperature a slow stream of dried air (KOH, H_2SO_4) was passed through the reaction mixture via an inlet tube while the reaction was followed by GC analyses. After 72 h chloroform was added to dilute the slurry and insoluble salts were filtered off using silica. The solvent was evaporated and the obtained brown oil was purified *via* vacuum flash chromatography using CH_2Cl_2:*Me*OH:NH_3 = 20:1:0.1 as eluent. Though it was not possible to separate d_{10}-anatabine **10** from the byproduct d_{10}-3,3′-bipyridine the yield of d_{10}-anatabine **10** (0.79 g corresponding to 4.8%) was determined by GC analyzes. GC: $R_t = 14.3$ min (d_{10}-anatabine), $R_t = 8.3$ min (d_{10}-3,3′-bipyridine).

1-Nitroso-2,2′,3′,4,4′,5,5′,6,6′,6′-decadeutero-1,2,3,6-tetrahydro-2,3′-bipyridine (d_{10}-N-Nitrosoanatabine) (**11**, $C_{10}HN_3OD_{10}$)

Compound **10** (1.86 g, 11.0 mmol) contaminated with d_{10}-bipyridine was dissolved in 16.4 cm^3 of 4 *N* HCl (65.6 mmol) and cooled to 0°C. A solution of 1.40 g (20.3 mmol) of $NaNO_2$ in 9 cm^3 of H_2O was added dropwise and the reaction mixture was stirred for 20 h at room temperature. Since there was still d_{10}-anatabine **10** left, according to TLC, another 0.65 g of $NaNO_2$ (9.4 mmol) in 4.5 cm^3 of H_2O were added at 0°C and the reaction was stirred for 3 h. A 2 *N* NaOH solution was added to adjust $pH = 14$

and the reaction mixture was extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The residue was purified by vacuum flash chromatography using CH_2Cl_2:*Me*OH:$NH_3 = 30:1:0.1$ as eluent followed by column chromatography to yield 103 mg (26%) yellow oil. ^{2}H NMR (61 MHz, $CCl_4 + 5\%$ d_4-*Me*OH): $\delta = 8.53$, 7.60, 7.30, 6.43, 6.10, 5.98, 5.84, 5.68, 5.02, 4.75 ppm; GC: $R_t = 17.7$ min; LC/UV: $R_t = 10.0$ min; LC/APCI-MS: $R_t = 4.4$ min, $m/z = 201$ $[M]^+$.

2,2′,3,4,4′,5,5′,6,6′6′-Decadeutero-3-(2-piperidyl)pyridine (d_{10}-Anabasine) (**12**, $C_{10}H_4N_2D_{10}$)

A solution of 1.90 g of d_{10}-anatabine **10** (0.6 mmol) contaminated with d_{10}-bipyridine in 250 cm^3 of anhydrous methanol was hydrogenated over 0.95 g of Pd/C at 3.5 bar for 3 h. The reaction mixture was filtered over silica and the solvent was evaporated under reduced pressure to yield 1.82 g of **12** as a brown oil. GC: $R_t = 13.9$ min (d_{10}-anabasine), $R_t = 8.4$ min (d_{10}-3,3′-bipyridine).

2,2′,3,4,4′,5,5′,6,6′,6′-Decadeutero-N-nitroso-3-(2-piperidyl)pyridine (d_{10}-N-Nitrosoanabasine) (**13**, $C_{10}H_3N_3OD_{10}$)

Compound **12** (1.82 g, 10.6 mmol) contaminated with d_{10}-bipyridine was dissolved in 16 cm^3 of 4 *N* HCl (65.6 mmol) and cooled to 0°C. A solution of 1.39 g (20.2 mmol) of $NaNO_2$ in 11 cm^3 of H_2O was added dropwise and the reaction mixture was stirred for 20 h at room temperature. Since there was still d_{10}-anabasine **12** left, according to TLC, another 0.39 g of $NaNO_2$ (5.7 mmol) in 3 cm^3 of H_2O were added at 0°C and the reaction was stirred for 4 h at room temperature. A 2 *N* NaOH solution was added to $pH = 14$ and the reaction mixture was extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The residue was purified by vacuum flash chromatography using CH_2Cl_2:*Me*OH:$NH_3 = 30:1:0.1$ as eluent followed by column chromatography to yield 117 mg (26%) of a yellow oil. ^{2}H NMR (61 MHz, $CCl_4 + 5\%$ d_4-*Me*OH): $\delta = 8.58$, 8.39, 7.67, 7.40, 6.33, 5.90, 4.8, 4.56, 3.65, 2.92, 2.6–1.5 ppm; LC/UV: $R_t = 11.9$ min; LC/APCI-MS: $R_t = 5.3$ min, $m/z = 203$ $[M]^+$.

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