Notes

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Nuclear Magnetic Resonance Spectra of N-Alkyl-N-(ω-carboxyalkyl)-nitrosamines and Their Esters, and Chromatographic Separation of the (*Z*)- and (*E*)-Conformers of the Esters

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Nuclear magnetic resonance (NMR) spectra of a series of N-alkyl-N-(ω-carboxyalkyl)-nitrosamines and their methyl or *p*-bromophenacyl esters have been obtained. The (*E*)- and (*Z*)-conformers of the esters were separated by high-pressure liquid chromatography (HPLC) and thin-layer chromatography. Conformer ratios of the N-nitrosamines and their esters were determined by NMR spectrometry and HPLC.

Keywords——bladder carcinogen; N-nitrosamine; N-alkyl-N-(ω-carboxyalkyl)nitrosamine; N-nitrosamino acid; NMR of N-nitrosamine; HPLC of N-nitrosamine; TLC of N-nitrosamine; (*E*)- and (*Z*)-conformers; separation of (*E*)- and (*Z*)-conformers

N-Butyl-N-(3-carboxypropyl)nitrosamine (BCPN) (Fig. 1, I: R=C_4H_9, R′=H, n=3), the principal urinary metabolite of N-butyl-N-(4-hydroxybutyl)nitrosamine as well as of N,N-dibutylnitrosamine, was demonstrated to be the carcinogenic proximate form of these N-nitrosamines to the urinary bladder of the rat.[2] In connection with our studies[3,4] on the metabolic fate of these compounds, a number of N-alkyl-N-(ω-carboxyalkyl)nitrosamines (I) related to BCPN were synthesized[5] in order to examine the relationship between chemical structure and the organotropic carcinogenicity to the urinary bladder. This paper reports nuclear magnetic resonance (NMR) spectral data for N-alkyl-N-(ω-carboxyalkyl)nitrosamines (I) and their methyl and *p*-bromophenacyl esters (II and III), and the separation of the (*Z*)- and (*E*)-conformers[6] of these esters by high-pressure liquid chromatography (HPLC) and by thin-layer chromatography (TLC). These studies were required for the characterization and identification of urinary metabolites encountered in our studies.[3,4]

Materials and Methods

Chemicals——N-Alkyl-N-(ω-carboxyalkyl)nitrosamines (I) and their *p*-bromophenacyl esters (III) were prepared according to the procedure reported earlier.[5] Methyl esters (II) were prepared in the usual way by methylating N-alkyl-N-(ω-carboxyalkyl)nitrosamines with diazomethane in ether. After removal of the solvent by evaporation, an oily residue was purified by column chromatography on silica gel (E. Merck AG) using hexane–ether–CH_2Cl_2 (8:3:2: or 4:3:2) as an eluting solvent. Methyl esters prepared in the

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2) M. Okada and E. Suzuki, *Gann*, **63**, 391 (1972); M. Okada, E. Suzuki, and Y. Hashimoto, *ibid.*, **63**, 637 (1972).
3) M. Okada, E. Suzuki, J. Aoki, M. Iiyoshi, and Y. Hashimoto, *Gann Monograph on Cancer Research.*, **17**, 161 (1975).
4) M. Okada and M. Ishidate, *Xenobiotica*, **7**, 11 (1977).
5) M. Okada, E. Suzuki, and M. Iiyoshi, *Chem. Pharm. Bull.*, **26**, 3909 (1978).
6) The (*Z*)- and (*E*)-system for specifying double bond conformers unambiguously is employed.

Table I. Methyl Esters of N-Alkyl-N-(ω-carboxyalkyl)nitrosamines

Methyl ester of	$ON-N(R_1)(R_2)$ R_1	R_2	Formula	Analysis (%) Calcd (Found) C	H	N
MCPN[5]	CH_3	$(CH_2)_3COOH$	$C_6H_{12}N_2O_3$	44.99 (45.29	7.55 7.73	17.49 17.75)
ECPN	C_2H_5	$(CH_2)_3COOH$	$C_7H_{14}N_2O_3$	48.26 (48.55	8.10 8.28	16.08 15.85)
PCPN	C_3H_7	$(CH_2)_3COOH$	$C_8H_{16}N_2O_3$	51.05 (51.04	8.57 8.64	14.88 14.90)
BCPN	C_4H_9	$(CH_2)_3COOH$	$C_9H_{18}N_2O_3$	53.44 (53.36	8.97 8.92	13.85 14.01)
ACPN	C_5H_{11}	$(CH_2)_3COOH$	$C_{10}H_{20}N_2O_3$	55.57 (55.38	9.32 9.38	12.95 12.86)
t-BCPN	*tert*-C_4H_9	$(CH_2)_3COOH$	$C_9H_{18}N_2O_3$	53.44 (53.46	8.97 9.25	13.85 14.13)
ECEN	C_2H_5	$(CH_2)_2COOH$	$C_6H_{12}N_2O_3$	44.99 (45.47	7.55 7.83	17.49 17.19)
PCEN	C_3H_7	$(CH_2)_2COOH$	$C_7H_{14}N_2O_3$	48.26 (48.48	8.10 8.24	16.08 15.88)
BCEN	C_4H_9	$(CH_2)_2COOH$	$C_8H_{16}N_2O_3$	51.05 (51.12	8.57 8.56	14.88 14.86)
ACEN	C_5H_{11}	$(CH_2)_2COOH$	$C_9H_{18}N_2O_3$	53.44 (53.69	8.97 9.15	13.85 13.91)
MCMN	CH_3	CH_2COOH	$C_4H_8N_2O_3$	36.36 (36.17	6.10 6.24	21.20 20.98)
ECMN	C_2H_5	CH_2COOH	$C_5H_{10}N_2O_3$	41.09 (41.27	6.90 6.97	19.17 18.97)
BCMN	C_4H_9	CH_2COOH	$C_7H_{14}N_2O_3$	48.26 (48.78	8.10 8.16	16.08 15.81)
ACMN	C_5H_{11}	CH_2COOH	$C_8H_{16}N_2O_3$	51.05 (51.24	8.57 8.65	14.88 14.89)
BHCPN	C_4H_9	$CH_2CH(OH)CH_2COOH$	$C_9H_{18}N_2O_4$	49.53 (49.81	8.31 8.39	12.84 12.88)

present work and their elemental analysis data are listed in Table I.[7]

Methods——NMR spectra were taken at 34° in 5% $CDCl_3$ at 60 MHz, using a Hitachi R-20A spectrometer. Freshly prepared solutions of the compounds were used for measurement. Chemical shifts are expressed in δ (parts per million) with tetramethylsilane as an internal standard: s, singlet; d, doublet; t. triplet; q, quartet.

HPLC measurements were performed on an Oriental Motor 55K25GK-A liquid chromatograph equipped with a 254-nm fixed-wavelength ultraviolet detector (model NS-301 of Nihon Seimitsu Co., Ltd., Tokyo). Chromatography of the compounds was studied on a column (25 cm × 4.6 mm I.D.) packed with LiChrosorb SI-60 (5 μm) (E. Merck AG). The eluting solvents used were: S_1, hexane–ether–CH_2Cl_2 (4: 3: 2); S_2, S_1–EtOH (100: 1); S_3, hexane–ether (1: 1); S_4, hexane–ether (3: 2). The flow rate was 2.3 ml/min and the column temperature was 25°.

TLC was performed on plates coated with a 0.25 mm layer of silica gel HF_{254} (E. Merck AG), using hexane–ether–CH_2Cl_2 (4: 3: 2) as a developing solvent. Spots were visualized with ultraviolet (UV) light (254 nm) and with the reagent reported earlier.[8]

7) The following abbreviations are used: MCPN, N-methyl-N-(3-carboxypropyl)nitrosamine; ECPN, N-ethyl-N-(3-carboxypropyl)nitrosamine; PCPN, N-propyl-N-(3-carboxypropyl)nitrosamine; BCPN, N-butyl-N-(3-carboxypropyl)nitrosamine; ACPN, N-amyl(=pentyl)-N-(3-carboxypropyl)nitrosamine; *t*-BCPN, N-*tert*-butyl-N-(3-carboxypropyl)nitrosamine; ECEN, N-ethyl-N-(2-carboxyethyl)nitrosamine; PCEN, N-propyl-N-(2-carboxyethyl)nitrosamine; BCEN, N-butyl-N-(2-carboxyethyl)nitrosamine; ACEN, N-amyl-N-(2-carboxyethyl)nitrosamine; MCMN, N-methyl-N-(carboxymethyl)nitrosamine; ECMN, N-ethyl-N-(carboxymethyl)nitrosamine; BCMN, N-butyl-N-(carboxymethyl)nitrosamine; ACMN, N-amyl-N-(carboxymethyl)nitrosamine; BHCPN, N-butyl-N-(2-hydroxy-3-carboxypropyl)-nitrosamine.

8) M. Okada, E. Suzuki, and M. Iiyoshi, *Chem. Pharm. Bull.*, **26**, 3891 (1978).

The compounds were dissolved in EtOAc or CH_2Cl_2 and the freshly prepared solutions were subjected to HPLC and TLC.

Results and Discussion

The partial double bond character of the N–N linkage in asymmetric N-nitrosamines leads to the formation of *syn*- and *anti*-conformers (Fig. 1). The conformers are generally distinguishable by means of NMR spectrometry.[9–13] There are several reports[14–16] dealing with NMR spectroscopy of the conformers of N-nitrosamines with a carboxymethyl group (I, $n=1$), but no report has appeared on N-nitrosamines having a longer ω-carboxyalkyl chain.

The NMR spectral data for N-alkyl-N-(ω-carboxyalkyl)nitrosamines (I) are given in Table II. Conformational assignment was carried out according to Karabatsos and Taller;[10] protons resonate at higher magnetic fields when *syn* than when *anti* to the nitroso oxygen. As shown in Table II, four protons at carbon atoms adjacent to the amino nitrogen atom resonated at 3.4—4.4 ppm; these comprised two protons *syn* to the nitroso oxygen at 3.4—3.8 ppm and those *anti* at 4.1—4.4 ppm, with the exception of two protons at the carbon atom of the carboxymethyl group in N-alkyl-N-(carboxymethyl)nitrosamines (I, $n=1$), the data for which are indicated again under $-C\underline{H}_2COOH$ in the table. The chemical shifts of the two protons at the carbon atom adjacent to the ω-carboxyl group were definitely affected by the alkyl chain length, showing downfield shifts with shortening, *i. e.*, with decrease in the number n. Furthermore, as indicated in the table, the difference ($\Delta\delta$) in the chemical shifts due to these two protons between (*Z*)- and (*E*)-conformers was appreciable among the compounds with $n=3$, 2, and 1.

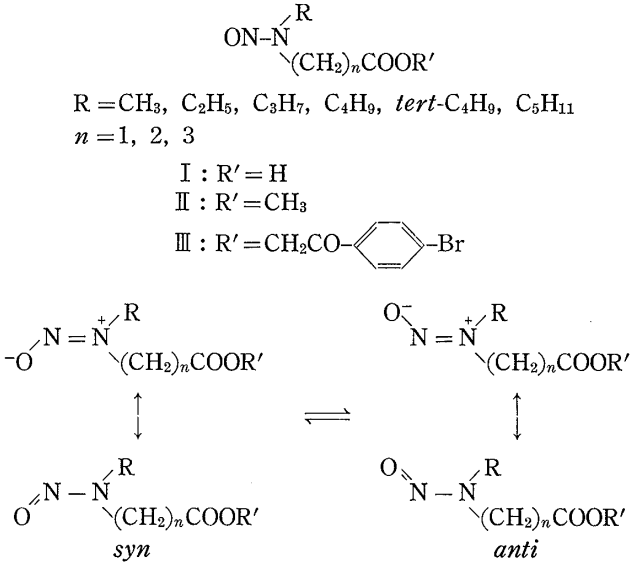

Fig. 1. N-Alkyl-N-(ω-carboxyalkyl)nitrosamines and Their Polar Resonance Forms

The designations *syn* and *anti* refer to the position of the nitroso group with respect to the ω-carboxyalkyl group.

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Table II. NMR Spectral Data for N-Alkyl-N-(ω-carboxyalkyl)nitrosamines and Their Conformer Ratios

Compound[7]	Chemical shifts,[a] δ ppm									Conformer ratio (%)	
	>N–CH₂– (Z)	(E)	Δδ[b] ×100	>N–(CH₂)ₙ– (Z)	(E)	Δδ ×100	–CH₂COOH (Z)	(E)	Δδ ×100	(Z)	(E)
MCPN				3.70	4.22(t)	52		2.30(t)	0	25	75
ECPN	4.17	3.63(q)	54	3.64	4.16(t)	52		2.37(t)	0	50	50
PCPN	4.09	3.55(t)	54	3.64	4.17(t)	53		2.38(t)	0	50	50[c]
BCPN	4.07	3.53(t)	54	3.59	4.12(t)	53		2.34(t)	0	50	50[c]
ACPN	4.10	3.55(t)	55	3.62	4.15(t)	53		2.35(t)	0	50	50[c]
t-BCPN				—	3.4—3.7		—	2.43(t)		0	100
ECEN	4.24	3.65(q)	59	3.80	4.37(t)	57	2.62	2.92(t)	30	55	45
PCEN	4.14	3.56(t)	58	3.78	4.34(t)	56	2.62	2.91(t)	29	60	40
BCEN	4.19	3.63(t)	56	3.81	4.37(t)	56	2.62	2.91(t)	29	62	38
ACEN	4.16	3.58(t)	58	3.78	4.34(t)	56	2.62	2.92(t)	30	60	40
MCMN				4.29	4.95(s)	66	4.29	4.95(s)	66	44	56
ECMN	4.29	3.69(q)	60	4.23	4.95(s)	72	4.23	4.95(s)	72	82	18
BCMN	4.24	3.67(t)	57	4.22	4.94(s)	72	4.22	4.94(s)	72	80	20
ACMN	4.22	3.63(t)	59	4.22	4.94(s)	72	4.22	4.94(s)	72	80	20
BHCPN	3.5—4.4			3.5—4.4			2.49	2.61(d)	12	50	50[c]

a) J=6.0—7.5 Hz.
b) Difference between the chemical shifts of the two conformers.
c) Estimated roughly from the peak heights of signals, but not by NMR integration of the peak areas.

The approximate conformer ratio was determined by integration of peak areas and is indicated in the same table. *t*-BCPN, which has a bulky *tert*-butyl group, existed exclusively as the (*E*) (*syn*)-conformer. N–Methyl-N-phenylnitrosamine, N-methyl-N-*tert*-butylnitrosamine[10] and N-*tert*-butyl-N-(4-hydroxybutyl)nitrosamine[13] were also found to exist only as the (*E*) (*syn*)-conformer. The (*E*) (*anti*)-conformer was the favored one for MCPN owing to the smallness of the methyl group in contrast to the opposing group. Both conformers existed in roughly equal ratios with compounds having the carboxypropyl group (I, n=3), while the (*Z*)-conformer was the preferred one for compounds with the carboxyethyl group (I, n=2), and it was also found to be the major conformer with compounds having the carboxymethyl group (I, n=1) except for MCMN, in which the (*E*)-conformer was predominant (as with MCPN).

The NMR spectral data and the approximate conformer ratios of the methyl esters (II) and *p*-bromophenacyl esters (III) of N-alkyl-N-(ω-carboxyalkyl)nitrosamines (I) are summarized in Table III. The chemical shifts of protons at the carbon atoms adjacent to the amino nitrogen and the carboxyl group in II and III did not differ appreciably (less than 0.2 ppm) from those observed with I (Table II), so that only the chemical shifts of the newly introduced protons, $-COOCH_3$ and $-CH_2CO-C_6H_4-Br$, are shown in the table. There are no differences in $\Delta\delta$ values between the corresponding II and III in regard to n, while an appreciable difference is observed within II or III.

The separation of the conformers of substituted asymmetric N-nitrosamines by HPLC has been reported recently.[13,17] The conformers of the methyl esters were separated by HPLC; the solvent system employed and the retention time (t_R, min) are shown in Table IV. The conformer ratio was determined from the peak heights and is also indicated in the table. By comparing the conformer ratios obtained for methyl esters of MCPN and ECPN by NMR spectrometry and HPLC, it can be judged that the (*Z*)-conformer has a shorter t_R than the (*E*)-conformer. Analogously, the faster eluting compound was regarded as the (*Z*)-conformer

17) W.T. Iwaoka, T. Hansen, S.-T. Hsieh, and M.C. Archer, *J. Chromatogr.*, **103**, 349 (1975).

Table III. NMR Spectral Data for Methyl and *p*-Bromophenacyl Esters of N-Alkyl-N-(ω-carboxyalkyl)nitrosamines and Their Conformer Ratios

Compound[7]	Chemical shifts, δ ppm: Methyl ester (II) –COOCH$_3$ (Z)	(E)	Δδ[a] × 100	*p*-Bromophenacyl ester (III) –CH$_2$CO–C$_6$H$_4$–Br (Z)	(E)	Δδ × 100	Conformer ratio (%): Methyl ester (Z)	(E)	*p*-Bromophenacyl ester (Z)	(E)
MCPN	3.68		0	5.30		0	24	76	24	76
ECPN	3.69		0	5.31		0	49	51	51	49
PCPN	3.69		0	5.32		0	50	50[b]	50	50[b]
BCPN	3.69		0	5.30		0	50	50[b]	50	50[b]
ACPN	3.68		0	5.32		0	50	50[b]	50	50[b]
t-BCPN		3.68		—	5.32		0	100	0	100
ECEN	3.69	3.72	3	5.32	5.35	3	56	44	60	40
PCEN	3.68	3.71	3	5.31	5.34	3	64	36	60	40
BCEN	3.69	3.72	3	5.30	5.33	3	63	37	61	39
ACEN	3.67	3.70	3	5.30	5.33	3	62	38	62	38
MCMN	3.82	3.74	8	5.33	5.42	9	49	51	46	54
ECMN	3.74	3.82	8		—[c]		79	21		
BCMN	3.71	3.80	9	5.32	5.41	9	77	23	76	24
ACMN	3.70	3.79	9	5.33	5.42	9	80	20	64	36
BHCPN	3.72		0	5.39		0	50	50[b]	50	50[b]

a) Difference between the chemical shifts of the two conformers.
b) Estimated roughly from the peak heights of signals, but not by NMR integration of the peak areas.
c) not synthesized

for the other carboxypropyl compounds (II, $n=3$) except in the case of *t*-BCPN, which showed a single peak, as expected from the NMR spectral data. With the carbomethoxyethyl compounds (II, $n=2$), comparison of the conformer ratio obtained by NMR spectroscopy with that determined by HPLC suggests that the (*Z*)-conformer is eluted faster than its counterpart, while the reverse relationship was observed with the carbomethoxymethyl compounds (II, $n=1$), namely the (*E*)-conformer was eluted earlier.

Separation of the conformers of the *p*-bromophenacyl esters by HPLC was performed similarly. As all the *p*-bromophenacyl esters were obtained in crystalline form, the conformer

Table IV. Separation of the (*Z*)- and (*E*)-Conformers of Methyl Esters of N-Alkyl-N-(ω-carboxyalkyl)nitrosamines by HPLC

Methyl ester of	Solvent system	Retention time (min) (Z)	(E)	Conformer ratio (%) (Z)	(E)
MCPN	S_1	3.9	4.3	27	73
ECPN	S_4	6.2	6.6	47	53
PCPN	S_4	4.3	4.6	50	50
BCPN	S_4	3.5	3.8	52	48
ACPN	S_4	3.1	3.4	52	48
t-BCPN	S_4		3.6	0	100
ECEN	S_1	2.4	2.9	58	42
PCEN	S_1	2.0	2.4	61	39
BCEN	S_1	1.8	2.1	59	41
ACEN	S_1	1.7	2.0	60	40
MCMN	S_1	3.2	2.8	48	52
ECMN	S_1	2.4	2.1	72	28
BCMN	S_3	2.4	2.0	76	24
ACMN	S_3	2.1	1.8	76	24
BHCPN	S_2	3.5	4.1	56	44

ratio was determined using freshly prepared ethyl acetate solutions of the esters or after equilibration of the conformers. The mobile phase used, retention time and conformer ratio are given in Table V. The retention times for both conformers were determined by comparing the conformer ratio obtained by NMR spectrometry (Table III) with that determined by HPLC. The (*Z*)-conformer showed a shorter t_R without exception among the *p*-bromophenacyl esters. As shown in the table, the (*E*)-conformer was predominant immediately after dissolution, but on standing in solution the (*Z*)-conformer became dominant as a result of conformational interconversion, except in the cases of MCPN and MCMN. It was found generally that a time of less than six hours was sufficient to attain equilibrium of the conformers when freshly prepared ethyl acetate solutions of the esters were allowed to stand at room temperature. In the case of the *p*-bromophenacyl ester of ACEN, for example, which existed almost exclusively as the (*E*)-conformer in the crystalline state, and thus also immediately after dissolution, it was shown by continuous scanning NMR measurement that equilibrium was attained after 80 min. The conformer ratios of the *p*-bromophenacyl esters, therefore, were determined by HPLC immediately after dissolution and after standing for 6 hr at room temperature.

Table V. Separation of the (*Z*)- and (*E*)-Conformers of *p*-Bromophenacyl Esters of N-Alkyl-N-(ω-carboxyalkyl)nitrosamines by HPLC

p-Bromophenacyl ester of	Solvent system	Retention time (min)		Conformer ratio (%) Immediately after dissolution		Conformer ratio (%) 6 hr after dissolution	
		(*Z*)	(*E*)	(*Z*)	(*E*)	(*Z*)	(*E*)
MCPN	S_1	4.4	5.1	8	92	22	78
ECPN	S_3	8.0	9.5	37	63	51	49
PCPN	S_3	5.7	6.6	31	69	55	45
BCPN	S_3	5.0	5.9	41	59	55	45
ACPN	S_3	4.4	5.2	26	74	54	46
t-BCPN	S_3		5.1	0	100	0	100
ECEN	S_1	4.3	5.8	3	97	63	37
PCEN	S_1	3.0	3.9	12	88	63	37
BCEN	S_1	2.2	2.7	5	95	62	38
ACEN	S_1	2.0	2.4	5	95	61	39
MCMN	S_3	8.4	9.5	43	57	46	54
BCMN	S_3	3.2	3.8	19	81	78	22
ACMN	S_3	2.9	3.5	24	76	64	36
BHCPN	S_2	4.2	5.8	35	65	60	40

Successful separations of the (*Z*)- and (*E*)-conformers of N-nitrosamines by TLC have appeared in the literature.[13,18,19] Of the three groups of compounds (I, II and III), the *p*-bromophenacyl esters (III) were most easily detectable as two spots on thin-layer chromatograms, while I and II generally appeared as single spots except for compounds with $n=2$, when a mixture of hexane, ether and dichloromethane was used as a solvent. Chromatograms obtained with freshly prepared solutions of III ($n=2$) showed a spot of the (*E*)-conformer with a lower *Rf* value as the principal spot. On standing at room temperature, however, all of the solutions showed a gradual increase in the upper spot area of the (*Z*)-conformer in accordance with the results obtained by HPLC.

The conformational stability of the rotational isomers of simple N,N-dialkylnitrosamines is largely governed by the steric requirements of the two substituents.[10] In the case of N-

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methyl-N-ethylnitrosamine, an equilibrium $(E)/(Z)$ ratio of about 70/30 is attained.[10] With the *p*-bromophenacyl ester of MCMN, the ester moiety is bulkier than ethyl, and thus the $(E)/(Z)$ ratio was about 60/40, showing that the (Z)-conformer is considerably stabilized relative to the (E)-conformer, possibly as a result of electrostatic or dipole interactions, as indicated in structure IV (Fig. 2).[15]

Fig. 2. Electrostatic or Dipole Interactions in the Structures of Esters of N-Alkyl-N-(ω-carboxyalkyl)nitrosamines

The equilibrium $(E)/(Z)$ ratios observed in the present work with the esters of carboxymethyl ($n=1$), carboxyethyl ($n=2$), and carboxypropyl ($n=3$) compounds were approximately 30—20/70—80, 40/60 and 50/50, respectively, almost regardless of the opposing alkyl group, except for compounds with a methyl or *tert*-butyl group. These results may be well explained by the contribution of structures IV and V for carboxymethyl and carboxyethyl compounds, respectively, while no contribution of a seven-membered structure derivable from carboxypropyl compounds would be expected.

The data on BHCPN (β-hydroxylated BCPN) are also included in the tables; this was isolated as a minor urinary metabolite of N-butyl-N-(4-hydroxybutyl)nitrosamine as well as of N,N-dibutylnitrosamine.[2]

The results described in this paper are valuable as a guide for the separation and identification of urinary metabolites and for determining their purity. In particular, the NMR spectral data are useful for determining the number of methylene groups ($n=1, 2, 3$) between the amino nitrogen and the carboxyl group in minor acidic metabolites of N,N-dialkylnitrosamines.[2]

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