Monatshefte für Chemie 128, 291–299 (1997)

Monatshefte für Chemie
Chemical Monthly

Investigations on the Stability of Bendamustin, a Cytostatic Agent of the Nitrogen Mustard Type, I. Synthesis, Isolation, and Characterization of Reference Substances

R. Gust* and **R. Krauser**

Institut für Pharmazie, Freie Universität Berlin, Königin-Luise-Straße 2 + 4, D-14195 Berlin, Germany

Summary. The following compounds were chosen as reference substances for HPLC investigations on 4-(6-*bis*(2-chloro-ethyl)amino-3-methylbenzimidazoyl(2))butyric acid (bendamustin), an antineoplastic agent of the N-lost type (synthesized or isolated from crude bendamustin): 4-(6-((2-chloroethyl)(2-hydroxyethyl)amino)-3-methylbenzimidazoyl(2))butyric acid (**HP1**), 4-(6-*bis*(2-hydroxyethyl)amino-3-methylbenzimidazoyl(2))butyric acid (**HP2**), ethyl-4-(6-*bis*(2-hydroxyethyl) amino-3-methylbenzimidazoyl(2))butyrate (dihydroxyester), and ethyl-4-(6-*bis*(2-chloroethyl) amino-3-methylbenzimidazoyl(2))butyrate (dichloroester). Furthermore, the so far unidentified side product 4-(7,8-dihydro-6-(2-chloroethylamino)-3-methyl-1,4-thiazino[3,2-g]benzimidazoyl(2))-butyric acid (**NP1**), formed in the last step of the synthesis, was isolated and identified.

Keywords. Bendamustin; Antineoplastic; Hydrolysis products; Reference substances; Spectroscopic characterization.

Untersuchungen zur Stabilität von Bendamustin, einem Cytostatikum vom N-Lost-Typ, 1. Mitt.: Synthese, Isolierung und Charakterisierung von Vergleichssubstanzen

Zusammenfassung. Die folgenden Verbindungen wurden als Vergleichssubstanzen für HPLC-analytische Untersuchungen von 4-(6-*Bis*(2-chlorethyl)amino-3-methylbenzimidazoyl(2))buttersäure (Bendamustin), einem Antitumormittel des N-lost-Typs, synthetisiert oder aus Bendamustin-Rohstoff vor der Endreinigung isoliert: (4-(6-((2-Chlorethyl)(2-hydroxyethyl)amino)-3-methylbenzimidazoyl(2))buttersäure (**HP1**), 4-(6-*Bis*(2-hydroxyethyl)amino-3-methylbenzimidazoyl(2))buttersäure (**HP2**), 4-(6-*Bis*(2-hydroxyethyl)amino-3-methylbenzimidazoyl(2))buttersäureethylester (Dichlorester). Weiterhin konnte das bislang unbekannte Nebenprodukt 4-(7,8-Dihydro-6-(2-chlorethylamino)-3-methyl-1,4-thiazino[3,2-*g*]benzimidazoyl(2))buttersäure (**NP1**), welches sich im letzten Schritt der Synthese bildet, isoliert und identifiziert werden.

Introduction

Substituted benzimidazoles are potent antagonists of amino acids and purines [1]. Depending on the substitution pattern, they inhibit the synthesis of proteins

and enzymes as well as the synthesis of nucleotides. Since tumor tissue needs high amounts of amino acids, benzimidazoles are suitable as antitumor agents. An enhancement of the tumor inhibiting properties can be achieved by combination with the cytotoxic N-lost moiety [2–6]. Decisive for antitumor as well as toxic side effects of these compounds is the basicity of the N-lost group.

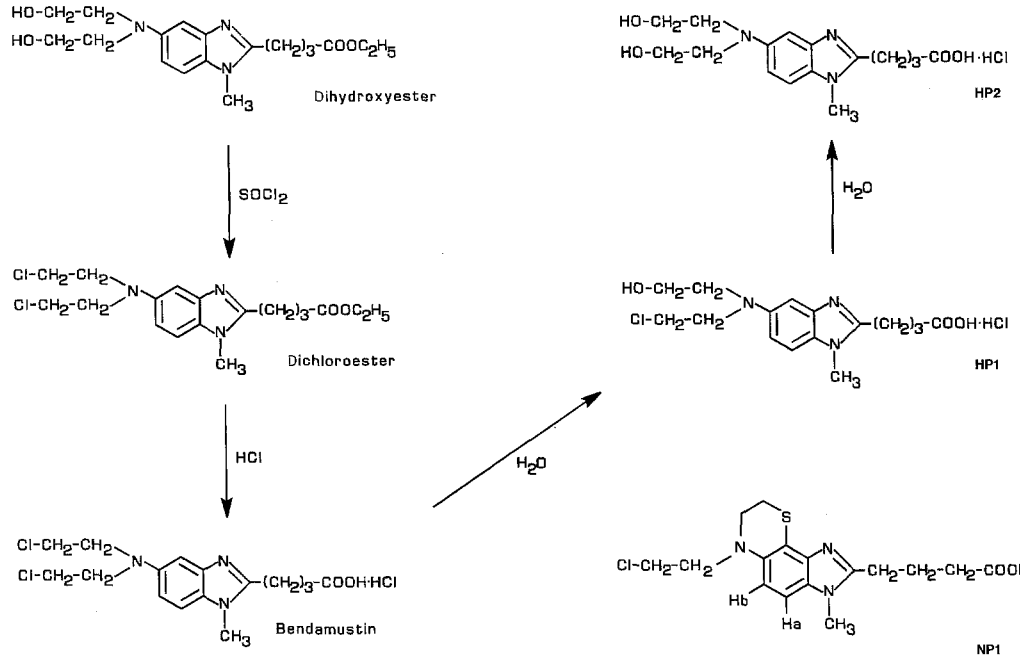
For derivatives of the 2-(*bis*(2-chloroethyl)aminomethyl)benzimidazole type, in which the CH_2 group prevents the influence of the heteroaromatic ring system on the basicity, strong toxic side effects have been predicted [4]. Therefore, for optimization of the pharmacological effects, the N-lost moiety was introduced in the 6-position of the benzimidazole ring. Additionally, position 3 was substituted with alkyl or aryl groups and position 2 with hydrophilic residues, *e.g.* aliphatic carboxylic acids. Among these compounds, bendamustin was found to be the most active one *in vivo* against several murine tumors [7–9]. In clinical tests, the cancerostatic effectiveness was confirmed for mammary carcinoma, lymphoma, and especially plasmocytoma [10–12]. Today, bendamustin (Ribomustin®) is a widely used chemotherapeutic agent, either alone or – more often – in combination with other antineoplastics, in the treatment of hematologic diseases and metastasized breast cancer.

Bendamustin is administered *iv* using a 0.9% NaCl solution. However, it must be considered that bendamustin hydrolyzes in water similar to other N-lost compounds. Recently, *Maas et al.* [13] have reported about the stability of the market drug product in aqueous NaCl solution (0.25 mg/ml, 0.9% NaCl solution; 4°C: $t_{90} = 120$h, 23°C: $t_{90} = 9$h; determined by the decrease of the bendamistin peak in HPLC). In addition to the characteristic bendamustin peak, the chromatograms exhibited further peaks which were empirically assigned, since no crystalline reference substances were available. In this paper we describe the synthesis or isolation as well as the characterization of the most important reference substances for the HPLC investigations of bendamustin.

Results and Discussion

Synthesis or isolation of bendamustin derivatives

The first synthesis of bendamustin has been performed by *Ozegowski et al.* [14] in an eleven step sequence starting from 2,4-dinitrochlorobenzene. The crucial conversions (Scheme 1) are the chlorination of ethyl 4-(6-*bis*(2-hydroxyethylamino)-3-methylbenzimidazoyl(2))butyrate (dihydroxyester) with $SOCl_2$ affording ethyl 4-(6-*bis*(2-chloroethyl)amino-3-methylbenzimidazoyl(2))butyrate (dichloroester) and the subsequent ester cleavage with HCl to obtain 4-(6-*bis*(2-chloroethyl)-amino-3-methylbenzimidazoyl(2))butyric acid (bendamustin). Under the reaction conditions employed, bendamustin hydrolyzes in small amounts to the hydroxychloro (**HP1**) and the dihydroxy derivative (**HP2**). For the HPLC analytical investigation of the drug substance and the market drug product Ribomustin®, the dihydroxyester, the dichloroester, and both hydrolysis products were chosen as suitable reference substances.



Scheme 1

Whereas the dihydroxyester and bendamustin were made available by courtesy of the Ribosepharm company, we synthesized the dichloroester from bendamustin by esterification in ethanolic HCl. **HP2** was obtained by quantitative hydrolysis of bendamustin in water as described by *Werner et al.* [15]. Since it was impossible to isolate **HP1** by fractional crystallization from an aqueous solution of bendamustin, MPLC on RP 18 was used for the separation. In this connection it was also possible to isolate the impurity detected by *Maas et al.* in Ribomustin® (**NP1**, [13]).

Characterization of bendamustin and its derivatives

Bendamustin and its derivatives were characterized by their elemental analyses, NMR, and mass spectra (Tables 1 and 2).

The ^1H NMR spectra exhibit a six spin system of the form AA′BB′CC′ for the butyric acid moieties with signals at $\delta = 2.92$–$3.22(CH^{\alpha})$, $\delta = 2.09$–2.15 (CH^{β}), and $\delta = 2.45$–$2.53(CH^{\gamma})$. The spin systems were approximatively interpreted following first order rules (see Table 2). It must be mentioned that – with exception of the dihydroxyester – the compounds were measured as their hydrochlorides. The positive charge leads to a low field shift for CH_3, H^a, H^b, and CH^{α}. Characteristic for bendamustin and its derivatives are the signals of the methylene groups $CH^{A/C}$ and $CH^{B/D}$. These protons afford an AA′BB′ system consisting of 12 badly resolved lines which again was interpreted using first order rules.

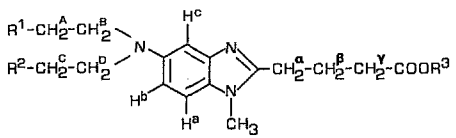
The $HO–CH_2–CH_2$ and $Cl–CH_2–CH_2–$ side chains allow an unequivocal assignment of bendamustin and its derivatives using the signals of $CH^{A/C}$ and $CH^{B/D}$ which are shifted about 0.10–0.15 ppm to lower field in the spectra of

Table 1. Analytical data of bendamustin and its derivatives

	M.p. °C	Formula	C		H		N		M+H[a]	
			calc.	found	calc.	found	calc.	found	calc.	found
Dihydroxyester	106–107	$C_{18}H_{27}N_3O_4$	61.87	61.82	7.79	7.83	12.03	11.99	350.2	350.3
Dichloroester	120–124	$C_{18}H_{25}Cl_2N_3O_2 \cdot HCl$	51.14	51.19	6.20	6.11	9.94	9.93	386.1	386.2
Bendamustin	165–166	$C_{16}H_{21}Cl_2N_3O_2 \cdot HCl \cdot 0.5H_2O$	47.60	47.63	5.70	5.66	10.41	10.40	358.1	358.1
HP1	160–162	$C_{16}H_{22}ClN_3O_3 \cdot HCl$	51.07	51.02	6.16	6.06	11.17	11.20	340.1	338.0
HP2	175–178	$C_{16}H_{23}N_3O_4 \cdot HCl$	53.71	53.82	6.76	6.72	11.74	11.73	322.2	322.2
NP1	182–187	$C_{16}H_{20}ClN_3O_2 \cdot S \cdot HCl \cdot 2.5H_2O$	44.14	43.94	5.40	5.89	9.66	9.64	354.1	354.4

[a] PILISI-FAB mass spectra in a methanol/glycerol matrix

Table 2. ^{1}H NMR data of bendamustin and its derivatives (250 MHz, methanol-d_4, *TMS*)



	R^1	R^2	R^3	$CH^A/CH^B/CH^C/CH^D$ (ppm)	$CH^\alpha/CH^\beta/CH^\gamma$ (ppm)	$H^a/H^b/H^c$ (ppm)	$N-CH_3$ (ppm)	$-CH_2-CH_3$ (ppm)
Dihydroxyester	OH	OH	Et	3.51 (t, ^3J = 6.2 Hz, $CH^{B/D}$)	2.09 (*quin*, ^3J = 7.4 Hz, CH^β)	6.97 (d, ^3J = 2.0 Hz, H^c)		
				3.72 (t, ^3J = 5.7 Hz, $CH^{A/C}$)	2.45 (*t*, ^3J = 6.9 Hz, CH^γ)	6.88, 6.91 (dd, ^3J = 2.4 Hz, 8.9 Hz, H^b)	3.73 (s)	1.20 (t, ^3J = 7.1 Hz, CH_2)
					2.92 (t, ^3J = 7.8 Hz, CH^α)	7.26 (d, ^3J = 8.7 Hz, H^a)		4.04 (q, ^3J = 7.1 Hz, CH_3)
Dichloroester	Cl	Cl	Et	3.70 (t, ^3J = 5.8 Hz, $CH^{B/D}$)	2.11 (*quin*, ^3J = 7.0 Hz, CH^β)	6.93 (d, ^3J = 2.4 Hz, H^c)		
				3.84 (t, ^3J= 6.0 Hz, $CH^{A/C}$)	2.51 (t, ^3J= 6.9 Hz,CH^γ)	7.10, 7.12 (dd, ^3J= 2.4 Hz, 9.4 Hz, H^b)	3.93 (s)	1.16 (t, ^3J = 7.1 Hz, CH_2)
					3.18 (t, ^3J = 8.0 Hz, CH^α)	7.65 (d, ^3J = 9.4 Hz, H^a)		4.00 (q, ^3J = 7.1 Hz, CH_3)
Bendamustin	Cl	Cl	H	3.75 (t, ^3J = 5.9 Hz, $CH^{B/D}$)	2.14 (quin, ^{3}J = 6.9 Hz, CH^β)	6.94 (d, ^3J = 2.3 Hz, H^c)		
				3.87 (t, ^3J = 5.7 Hz, $CH^{A/C}$)	2.53 (t, ^3J = 6.9 Hz, CH^γ)	7.13, 7.16 (dd, ^3J = 2.3 Hz, 9.2 Hz, H^b)	3.97 (s)	
					3.22 (t, ^3J = 8.0 Hz, CH^α)	7.67 (d, ^3J = 9.2 Hz, H^a)		
HP1	Cl	OH	H	3.65 (t, ^3J = 5.8 Hz, CH^D)	2.15 (br, CH^β)	6.98 (d, ^3J = 1.7 Hz, H^c)		
				3.86 (t, ^3J = 6.0 Hz, CH^A)	2.53 (t, ^3J = 6.5 Hz, CH^γ)	7.15, 7.18 (dd, ^3J = 1.7 Hz, 9.2 Hz, H^b)	3.97 (s)	
				3.73–3.76 (m, 4H, $CH^{B/C}$)	3.22 (t, ^3J = 7.6 Hz, CH^α)	7.64 (d, ^3J = 9.2 Hz, H^a)		
HP2	OH	OH	H	3.62 (t, ^3J = 5.9 Hz, $CH^{B/D}$)	2.12 (quin, ^3J = 7.1 Hz, CH^β)	6.90 (d, ^3J = 2.2 Hz, H^c)		
				3.76 (t, ^3J = 5.9 Hz, $CH^{A/C}$)	2.51 (t, ^3J = 6.7 Hz, CH^γ)	7.11, 7.15 (dd, ^3J = 2.3 Hz, 9.3 Hz, H^b)	3.94 (s)	
					3.19 (t, ^3J = 8.0 Hz, CH^α)	7.57 (d, ^3J = 9.3 Hz, H^a)		
NP1				3.17–3.23 (m, 2H)	2.09 (quin, ^3J = 7.0 Hz, CH^β)	7.12 (^3J = 9.2 Hz, H^b)	3.96 (s)	
				3.78–3.87 (m, 6H)	2.51 (t, ^3J = 6.6 Hz, CH^γ)	7.40 (^3J = 9.2 Hz, H^a)		
					3.20 (t, ^3J = 7.1 Hz, CH^α)			

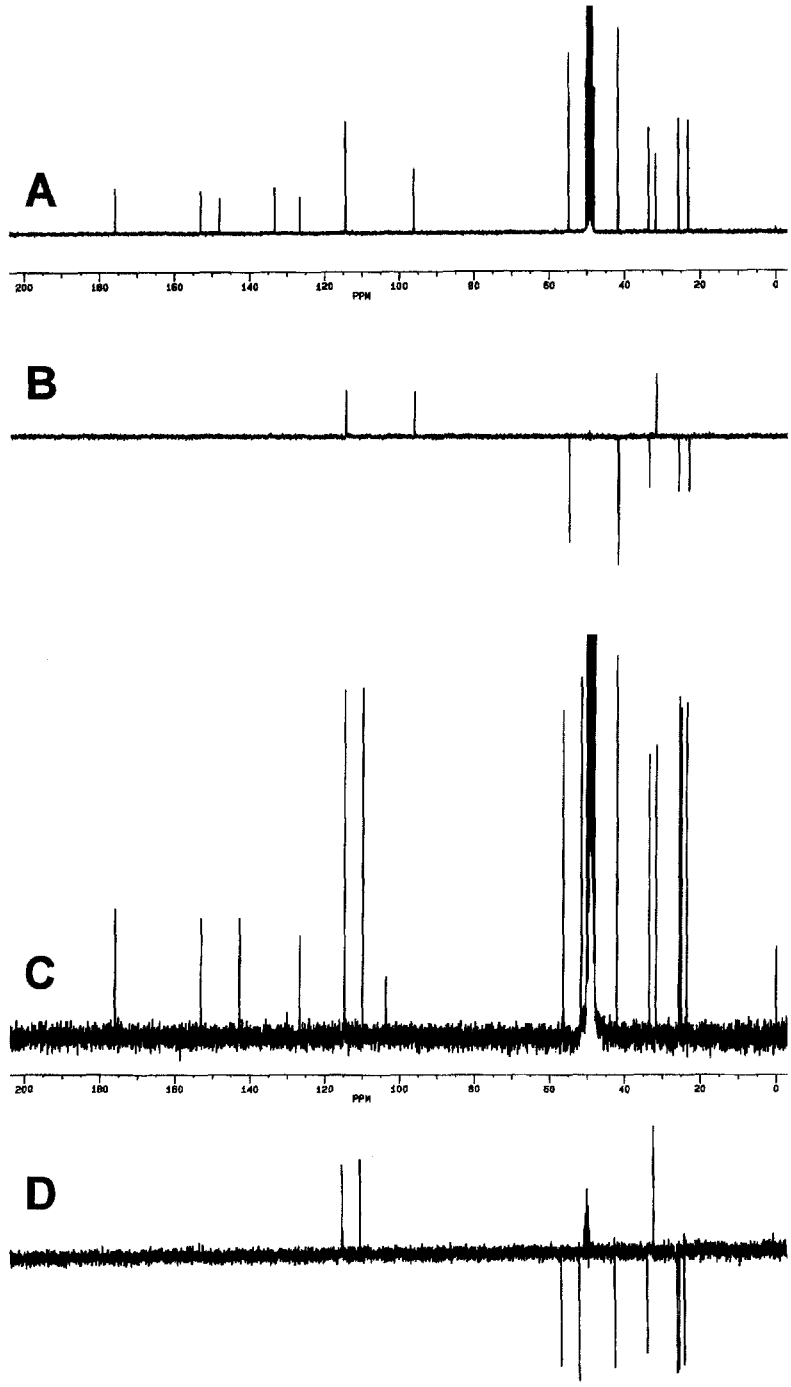
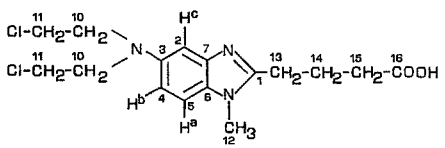


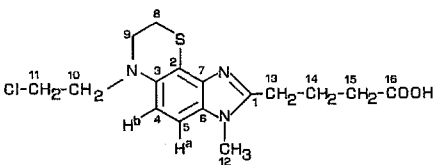
Fig. 1. ^{13}C NMR spectra (CPD, DEPT) of bendamustin (**A**, **B**) and **NP1** (**C**, **D**); methanol-d_4, *TMS*

bendamustin and its dichloroester. The spectrum of **HP1** exhibits the signals of both the HO–CH_2–CH_2–N and the Cl–CH_2–CH_2–N group: the chemical shifts of CH^A and CH^B are unchanged compared with bendamustin, whereas CH^C and CH^D are high field shifted as it was found for **HP2**. Together with the results from

Table 3. ^{13}C NMR data[a] of bendamustin and **NP1** (62.9 MHz, methanol-d_4, ppm, *TMS*)



Bendamustin



NP1

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
Bendamustin	152.9	96.1	148.0	114.4	114.5	126.5	133.3			41.6	54.7	31.7	25.6	23.0	33.5	175.8
NP1	153.1	103.5	142.8	114.7	109.8	126.6	130.0	25.0	51.5	42.0	56.2	31.6	25.5	23.5	33.4	175.9

elemental analyses (Table 1) and mass spectrometry, the chemical structures of bendamustin, its dihydroxyester, and its dichloroester as well as those of the hydrolysis products **HP1** and **HP2** were confirmed.

The side product **NP1** is formed in the last step of the synthesis (ester cleavage with HCl). The isolation from the crude product was achieved using MPLC. For the structural analysis, the ^{13}C NMR spectra of bendamustin and **NP1** were used (Fig. 1).

The assignment of the peaks were performed *via* a DEPT experiment which allows the distinction of signal multiplicities [16]. Fig. 1A shows the decoupled ^{13}C NMR spectrum of bendamustin together with the corresponding DEPT experiment. Together with an estimation of chemical shifts using an increment system [17], all signals of bendamustin could be assigned (Table 3).

In the ^{13}C spectrum of **NP1** the signals of C10 to C16 exhibit shift values comparable to those of bendamustin; therefore, an intact butyric acid moiety as well as the presence of an N-CH_3 group and at least one Cl–CH_2–CH_2–N side chain can be assumed. Among the aromatic C atoms, especially the signal of C2 is changed. Besides a low field shift of 7.4 ppm, the DEPT experiment indicates its conversion into a quaternary C-atom. Furthermore, the signals at $\delta = 25.0$ and 51.5 can be identified in the DEPT experiment as CH_2 groups. The resonance positions are very similar to those found in 1,4-thiazine ($\delta = 28.3$ and 47.9); therefore, cyclization of an N–CH_2–CH_2 chain via an S bridge to C2 can be assumed. This reduces the multiplicity of the aromatic protons in the ^1H NMR spectrum to an AB system with a coupling constant of 9.2 Hz (see Table 2). The proposed structure is also in accordance with the elemental analysis and the results of mass spectrometry (Table 1).

Experimental

^1H NMR spectra (250 MHz) and ^{13}C NMR spectra (62.9 MHz): PFT NMR spektrometer WM 250 (Bruker); mass spectra: Finnigan MAT 95A, PILISI-FAB in a methanol/glycerol matrix; MPLC: Gradientenformer Labomat VS200, MPLC pump MD-80/100, Labocol Roto-Ultra/120, Fa. Kronlab; FPGC-ODS4-S-120-S-15/30 MPLC precolumn (26 × 313 mm) und MPLC main column (37 × 539 mm).

Separation of bendamustin analogs by MPLC

Crude bendamustin (150 mg) was dissolved in 2 ml of a mixture of methanol/1 *N* HCl (1:1), loaded onto the MPLC column, and eluted with a mixture of methanol/water (1:1) which was adjusted to $pH = 3$ with 1 *N* HCl. From the separated fractions, the methanol was removed *in vacuo* and the resulting aqueous solutions were freeze dried. The derivatives eluted from the column in the following series: **HP2**, **HP1**, **NP1**, bendamustin, and dichloroester.

Syntheses

The syntheses of bendamustin and its hydrolysis product **HP2** were carried out according to *Ozegowski et al.* [4] and *Werner et al.* [15].

4-(6-Bis(2-chloroethyl)amino-3-methylbenzimidazoyl(2))ethyl butyrate (dichloroester)

Bendamustin (1 g, 2.48 mmol) was dissolved in 20 ml of ethanol and treated with gaseous HCl for 20 min. After 4h stirring at room temp., the solvent was evaporated and the crude dichlorester was recrystallized from ethanol to give a white powder; yield: 890 mg (85%).
For analytical data see Tables 1–3.

Acknowledgements

This work was supported by the *Fonds der Chemischen Industrie* and the Ribosefarm company. The technical assistance of *S. Bollwein* and *M. Kornau* is gratefully acknowledged. *R.G.* thanks *Werner Roth* for helpful discussions and critical reading of the manuscript.

References

[1] Kühnau J (1956) Arch Exp Path Pharmacol **228**: 87
[2] Knobloch W (1958) Chem Ber **91**: 2557
[3] Hirschberg W, Gellhorn A, Gump WS (1957) Cancer Res **17**: 904
[4] Ozegowski W, Krebs D (1971) Zbl Pharm **110**: 1013
[5] Rintelen K, Knobloch W (1958) Acta Biol Med German **1**: 109
[6] Ultmann JE, Thompson HG, Hirschberg E, Zeidenweber J, Gellhorn A (1959) Cancer Res **19**: 719
[7] Anger G, Hesse P, Köhler P, Baufeld H (1967) Dtsch Gesundheitsw **22**: 1099
[8] Jungstand W, Gutsche W (1967) Monatsber Dtsch Akad Wiss **9**: 335
[9] Schnabel R, Jungstand W, Gutsche W, Grimm H, Fritsch S (1967) Acta Biol Med German **19**: 543
[10] Hesse P, Jaschke E, Anger A (1972) Dtsch Gesundheitsw **27**: 2058
[11] Brockmann B, Geschke E, Schmidt UM, Ebeling K (1991) Geburtsh u Frauenheilk **51**: 383
[12] Herold M, Anger G, Höche D, Kästner D (1987) Med Klin **82**: 345
[13] Maas B, Huber C, Krämer I (1994) Pharmazie **49**: 775
[14] Ozegowski W, Krebs D (1963) J Prakt Chem IV, **20**: 178
[15] Werner W, Letsch G, Ihn W (1987) Pharmazie **42**: 272
[16] Doddrell DM, Pegg DT, Bendall MR (1982) J Magn Reson **48**: 323
[17] Kalinowski HO, Berger S, Braun S (1984) ^{13}C-NMR-Spektroskopie. Thieme, Stuttgart

Received August 26, 1996. Accepted (revised) October 21, 1996.