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

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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.

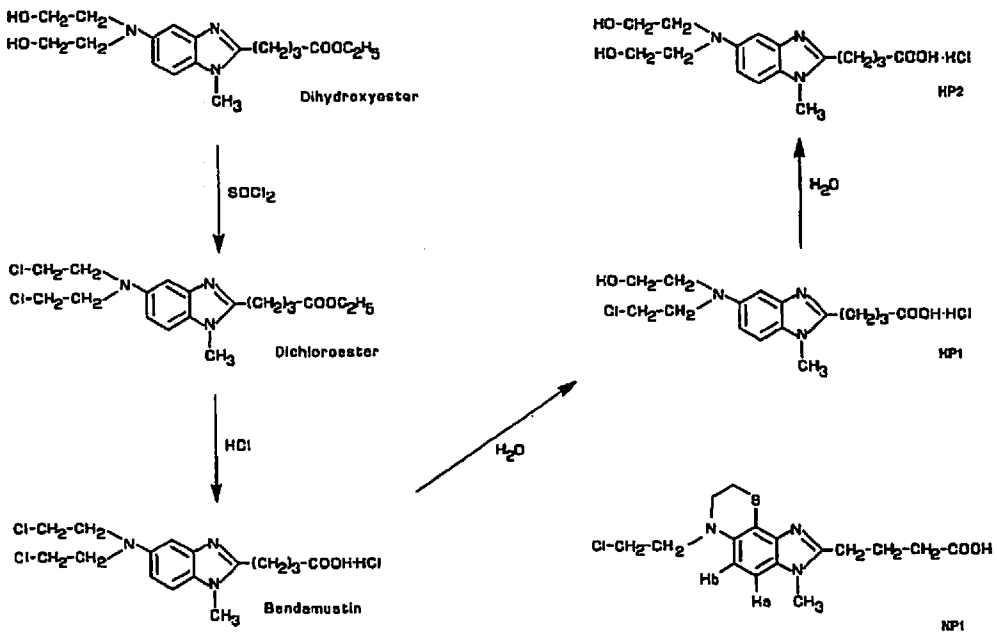
For derivatives of the 2-(*bis*(2-chloroethyl)aminomethyl)benzimidazole type, in which the CH_2 group prevents the influence of the heteroaromatic ring system on the basicity, strong toxic side effects have been predicted [4]. Therefore, for optimization of the pharmacological effects, the N-lost moiety 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 ^{1}H 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.

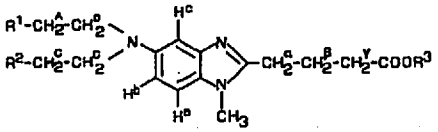
The HO–CH_2–CH_2 and Cl–CH_2–CH_2– side chains allow an unequivocal assignment of bendamustin and its derivatives using the signals of $CH^{A/C}$ and $CH^{B/D}$ which are shifted about 0.10–0.15 ppm to lower field in the spectra of

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 (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
					2.92 (t, ^3J = 7.8 Hz, CH^α)	7.26 (d, ^3J = 8.7 Hz, H^a)		4.04 (q, ^{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
					3.18 (t, ^3J = 8.0 Hz, CH^α)	7.65 (d, ^3J = 9.4 Hz, H^a)		4.00 (q, ^{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)			

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