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Synthesis and Preliminary Antitumor Evaluation of 4-(SR)-Sulfido-Cyclophosphamides

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Summary. *Crystalline 4-(SR)-sulfidocyclophosphamides, sulfido derivatives of activated cyclophosphamide (4-hydroxycyclophosphamide), were synthesized by ozonation of cyclophosphamide and reaction of the intermediate 4-hydroxycyclophosphamide with various thiols (HSR). The products were characterized by elemental analysis, ^1H NMR and IR spectroscopy, and mass spectrometry. ^1H NMR and polarimetric analysis demonstrated that they consist of racemic cis-isomers that are stable in the crystalline state at room temperature. In aqueous solution these derivatives are hydrolyzed to 4-hydroxycyclophosphamide and the corresponding thiol, with half-lives ranging between 4 and 17 min at 37° C and pH 7. The cytotoxicity of 4-(S-ethyl)- and 4-(S-ethanol)-sulfidocyclophosphamide against Yoshida sarcoma ascites cells and the toxicity in rats were found to be practically identical with those of activated cyclophosphamide. A preliminary evaluation of the curative effect after a single IV injection of 4-(S-ethane)- and 4-(S-ethanol)-sulfidocyclophosphamide in rats bearing Yoshida ascites sarcoma or of 4-(S-ethanol)-sulfidocyclophsphamide in nu/nu mice bearing human breast carcinoma xenografts suggested that these sulfido derivatives possess the same oncostatic efficacy as activated cyclophosphamide itself.*

Introduction

Cyclophosphamide (CP) and other anticancer agents of the *N*-(2-chloroethyl)-amido-oxazaphosphorinane series need enzymatic activation to develop alkylating and oncostatic properties [3]. In previous communications [10, 4] we have demonstrated that only 4-hydroxycyclophosphamide (4-OHCP), the primary product of enzymatic activation of CP by 'mixed function' oxidases, exhibits as high a cancerotoxic selectivity in vivo as the latent drug CP itself, whereas other CP metabolites were shown to be either highly toxic, like *N*-mustard phosphorodiamidic acid (NPDA), or nontoxic, like 4-ketocyclophosphamide (4-ketoCP), or to possess only weak alkylating and cytotoxic properties, like carboxy-phosphamide. The relatively high therapeutic indices (TIs) of 4-OHCP and of other 4-hydroxylated *N*-(2-chloroethyl)-amido-oxazaphosphorinanes [4] were shown by us to be based on special chemical reactions of the activated *N*-(2-chloroethyl)-amido-oxazaphosphorinanes that led to control of their alkylating and cytotoxic activity [7, 13]. Thus we found that by a reversible reaction with the SH groups of thiols and biomolecules the spontaneous breakdown of activated *N*-(2-chloroethyl)-amido-oxazaphosphorinanes to alkylating and cytotoxic metabolites can be controlled, being dependent on the actual concentration of the reactants [15, 7].

Besides the interest deactivation of 4-OHCP by thiols may find with respect to the mechanism af action of CP, it offers an opportunity to synthesize 4-(SR)-sulfidocyclophosphamides (4-(SR)-sulfidoCP) as stabilized forms of activated CP. These compounds may replace the latent drug in special situations of clinical chemotherapy, either where metabolic activation cannot proceed, e.g., during regional perfusion and local instillation into body cavities, or when as a result of liver malfunction enzymatic activation is the limiting factor for chemotherapy with this class of drugs. Activated CP (4-OHCP) itself cannot fulfill those demands, because of its high instability.

Since our previous studies revealed the suitability of 4-(SR)-sulfidocyclophosphamides [15, 14] in this context, we now report on their synthesis and give a preliminary evaluation of their biological properties.

Materials and Methods

All reagents, solvents and chromatography materials, unless otherwise noted, were products of E. Merck, Darmstadt, FRG. CP was a gift from ASTA-Werke AG, Brackwede, FRG.

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IR measurements were recorded with a Perkin-Elmer (Überlingen, FRG) spectrophotometer Model 257, and ^1H NMR spectra at 90 MHz with a Bruker (Karlsruhe-Forchheim, FRG) HX 90 instrument equipped with a Fourier transform (FT) attachment and a Nicolet (Offenbach/Main, FRG) FT 1083 computer. The spectra were obtained by the FT mode (8 K data points) at a probe temperature of 35° C, with tetramethylsilane (TMS) as an internal standard in d_6-DMSO.

Assignments of the proton absorptions of I–IV were suggested on the basis of the ^1H NMR analyses of CP [8] 4-OOHCP, and 4-OHCP [20] assignments of the proton absorptions of the sulfido groups in I and II were confirmed by spin decoupling.

Optical activity of I–IV (10 mg/1 ml $CHCl_3$ 'Uvasol') was measured with a Perkin-Elmer polarimeter Model 141 at 589, 578, 546, 436 and 365 nm at 22° C, 1-ml microcuvette, 1 = 100 mm, being used. The melting points, obtained with a melting point microscope (C. Reichert, Vienna), are uncorrected.

High-performance liquid chromatography (HPLC) was carried out with Waters (Königstein/Ts, FRG) equipment consisting of a pump, model M-6000A, universal injector U6K, μBondapak C_{18} column with inner diameter 7.9 mm and length 300 mm, and a differential refractometer (R 401) for compound detection (attenuation range × 64 for preparative and × 4 for analytical working) in combination with a BD 12 Integrating Recorder (Kipp & Zonen, Schönberg/Ts., FRG) running at 2 cm/min scan speed and with a 12-s time constant. Unless otherwise noted, a mixture of MeOH/H_2O (2 : 3) at a flow rate of 9 ml/min was used as solvent after suction through a teflon filter (LS 5.0 µm, Millipore, Neu-Isenburg, FRG).

Analytical thin layer chromatography (TLC) was performed on either aluminium sheets or glass plates with silica gel 60 F_{254}, 20 × 20 cm, with a layer thickness of 0.2 mm, while preparative separations (PLC) were performed analogously with 20 × 20 cm plates, with a layer thickness of 2 mm. Visualization was achieved by means of an NaN_3/iodine/starch spray for sulfur-containing compounds [5], an NBP spray (4-(4'-nitrobenzyl)-pyridine, EGA-Chemie, Steinheim, FRG) [9, modified] for alkylators, and exposure to iodine vapor, showing the different compounds as white or brown spots. All the reported R_f values are approximate.

Syntheses

2-[Bis(2-chloroethyl)amino]-2-oxo-4-hydroxy-1,3,2-oxazaphosphorinane (4-hydroxycyclophosphamide, 4-OHCP) was prepared via 4-hydroperoxycyclophosphamide (4-OOHCP), by ozonation of CP in H_2O/H_2O_2 at 0° C, as described previously [15], and subsequent deoxygenation with triphenylphosphin in CH_2Cl_2 at 0° C [20] to yield 4-OHCP as an oil, which was used without purification for the synthesis of the 4-(SR)-sulfidocyclophosphamides (I–IV).

Most of the 4-OOHCP preparations used for deoxygenation contained 10%–20% of 4-ketocyclophosphamide (4-ketoCP), as was obvious on HPLC. This 4-ketoCP, a by-product of 4-OOHCP synthesis (Fig. 1), can be removed from I–IV more easily than from 4-OOHCP or 4-OHCP by fractionated crystallization from diethylether/CH_2Cl_2 (1 : 1) at 0° C, at which temperature I–IV remain in solution.

Reaction of 4-OHCP with thiols to 4-(SR)-sulfidocyclophosphamides (I–IV): General Procedure. Reaction of 0.36 mmol 4-OHCP[1] in 2 ml CH_2Cl_2 was attained during stirring by dropwise addition of a solution containing 0.72 mmol of the thiol (HSR) and 5 mg trichloroacetic acid or 3 µl trifluoroacetic acid in 1 ml CH_2Cl_2. While ethanethiol, α-toluenethiol (benzylmercaptan), and 2-mercaptoethanol reacted at 0° C with 4-OHCP nearly quantitatively within 1 h, the reaction with 2-methyl-2-propanethiol (*tert*-butylmercaptan) took

1 The concentration of 4-OHCP in the oily residue from reduction, when containing 4-ketoCP, was determined by HPLC and/or by a fluorometric test [25]

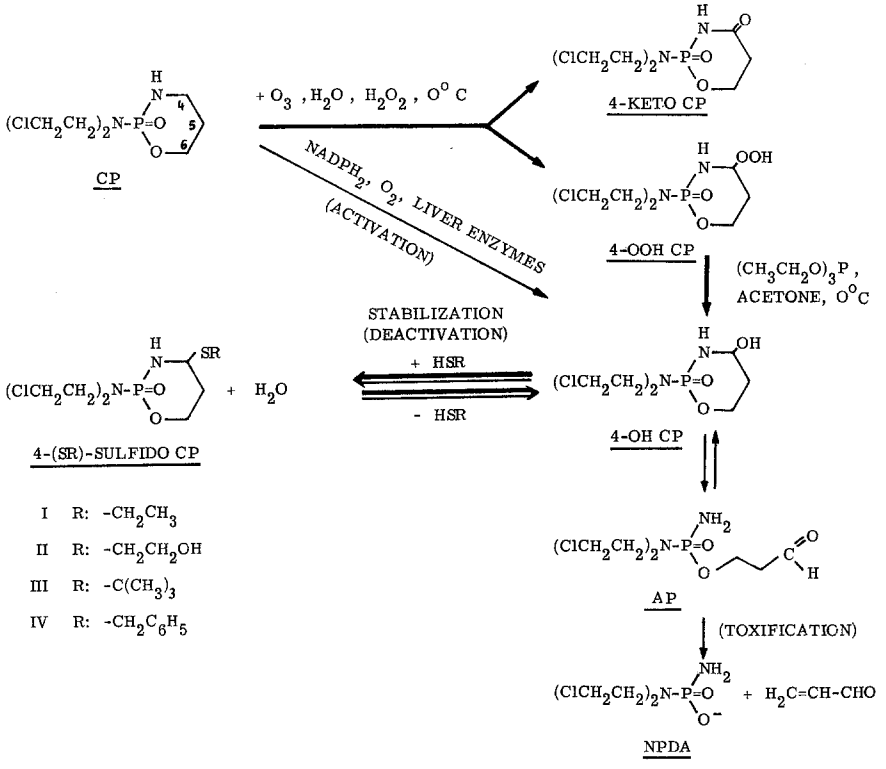

Fig. 1. Routes to 4-(SR)-sulfidocyclophosphamides (— synthetic and —— metabolic). In vivo HSR refer to HS biomolecules (e.g., glutathion, proteins)

Table 1. Physical and chemical data on 4-(SR)-sulfidocyclophosphamides

Compound	Yield from synthesis (%)	mp (°C)	Mol. wt. (calcd.)	^{1}H NMR-coupling const.[a] (Hz)		TLC on silicagel[b]		Solubility[c] in H_2O, 20° C (%)	Half-life in H_2O pH 7, 37° C (min)	pK_a of thiol involved[d]	Stability of the cryst. compds.[e, g]	
				$J(C_4\text{-H}, C_5\text{-H})$	$J(P, C_4\text{-H})$	Solvent[f]	R_f				−20° C	Room temp.
4-(S-ethyl)-sulfidoCP, I	74	53– 54	321.21	4.0	21	EA	0.43 (0.08)	1.5	4	10.50	m	h
4-(S-ethanol)-sulfidoCP, II	78	78– 79	337.21	4.6	21.5	EA/AC 1 : 1	0.58 (0.32)	9	7	9.50	no dec.	m
4-(S-*tert*-butyl)-sulfidoCP, III	61	89– 90	349.26	4.7	18.5	EA	0.55 (0.19)	< 0.1	17	11.22	no dec.	m
4-(S-benzyl)-sulfidoCP, IV	76	53– 54	383.28	4.3	21.7	EA	0.61 (0.30)	< 0.1	6	9.43	no dec.	m
4-OHCP	–	47– 48	277.10	3.3	22	EA/AC 4 : 1	0.19	> 10	–	–	d	min
4-OOHCP	–	109–110	293.10	3	24.5	EA	0.56	1.5	–	–	no dec.	w

[a] ^{1}H NMR-coupling constants listed, which became obvious after addition of D_2O, were used for structural predictions according to their dependence on the dihedral angle of the atoms indicated [12]. Accordingly, the compounds listed here are *cis*-isomers [15, 22, and references there]. This finding is supported by IR studies on II, which indicate the existence of an intramolecular bridge bond between the OH- and P=O groups (see footnote 4), which would only be possible for this structure. Optical inactivity, shown by all compounds, supports their existence as racemates

[b] R_f values in ( ) refer to the diastereomeric forms. I-IV were run at +4° C, 4-OHCP and 4-OOHCP at –20° C.

[c] Determined from saturated solutions with a fluorometric test [25] and an NBP test [9]

[d] Data from [19]

[e] Stability of the compounds exposed to the atmosphere was observed by means of TLC and HPLC, and in their melting points. Decomposition of the compounds was indicated by yellowing

[f] EA, ethylacetate; AC, acetone

[g] m, months; w, weeks; d, days; no dec., decomposition

several hours and was accompanied by partial decomposition of 4-OHCP to NPDA (see Fig. 1). This could be partially suppressed by running this reaction at −20° C. After spraying with NBP and sulfur reagents, TLC of the reaction mixtures showed a main spot with a high R_f corresponding to the compounds I–IV described below, and also a minor spot with a lower R_f (see Table 1).

The slow-migrating compounds were not crystallizable and were therefore separated by PLC at 0° C under conditions like those used for TLC (see Table 1). They could be obtained only as oils and were unstable at room temperature. Behavior on TLC and field-desorption mass spectrometry (FD-MS) characterized them as diastereomeric forms of I–IV [17].

I–IV were isolated and crystallized as described below after washing of the reaction mixtures at 0° C with 2 × 0.5 ml of a 0.1 *N* $NaHCO_3$ solution and with 1 × 0.5 ml H_2O and drying of the organic phase over Na_2SO_4 followed by concentration of it to an oil in vacuo at 0° C. For larger-scale preparations all quantities were multiplied.

2-[Bis(2-chloroethyl)amino]-2-oxo-4-sulfidoethyl-1,3,2-oxazaphosphorinane (4-(S-ethyl)-sulfidoCP), I. The oily residue from the reaction of 3 mmol 4-OHCP and 6 mmol (444 µl) ethanethiol was applied to four PLC plates at 4° C and run with ethylacetate/acetone (95 : 5, v/v) at 4° C over 4 h. The zone containing I was detected by spraying indicator strips with sulfur spray. The silica gel was scratched out and eluted with acetone at 4° C. The eluate was sucked through a teflon filter (5 µm, Millipore), dried over Na_2SO_4, and concentrated in vacuo with ice-cooling to an oily residue. After the addition of 1 ml diethylether and 3 ml petroleum ether 40–60, crystallization was induced by scratching on the flask with a glass rod and subsequent placing on dry ice. If an oily bottom layer appeared, the flask was warmed to room temperature and some drops of CH_2Cl_2 were added. Warming to 20° C and cooling on dry ice was repeated several times until the first colorless needles were detected. For complete crystallization the flask was kept at −20° C overnight. Crystals were sucked off at 4° C and washed with −20° C cold petroleum ether 40–60. By this means, 0.79 g (82%) crude I was obtained, with a melting point (mp) of 30°–38° C. Recrystallization from CH_2Cl_2/diethylether/petroleum ether, a similar procedure to the one described above, yielded 0.71 g (74%) pure I, mp 53°–54° C, ir $\frac{KBr}{max}$ (cm^{-1}): 3185 (NH), 2975, 1455, 1425, 1365, 1265, 1215 (P = O), 1125, 1070 (POC), 985, 940, 880, 855, 745 (CCl); ^1H NMR (TMS, DMSO-d_6) δ 1.17 (t, $J_{SCH_2,\ SCH_2CH_3}$ = 7.3 Hz, SCH_2CH_3, 3 H), 1.53–2.33 (m, C_5–H_2, 2 H), 2.33–2.92 (m, $J_{SCH_2,\ SCH_2CH_3}$ = 7.3 Hz, SCH_2 + DMSO), 2.92–3.86 (m, $2CH_2CH_2Cl$, 8 H), 3.86–4.43 (m, C_6-H_2, 2 H), 4.68 (d of m, $J_{C4\text{-}H,\ P}$ = 21 Hz, C_4-H, 1 H), 5.39 (d of d, $J_{NH,P}$ = 6.7 Hz, $J_{NH,C4-H}$ = 4.7 Hz, NH, 1H). On deuterium exchange δ 5.39 disappeared and d of m δ 4.68 degenerated to d of t, $J_{C4-H,C5-H_2}$ = 4 Hz. Anal. ($C_9H_{19}N_2O_2SPCl_2$) calcd.: C 33.65; H 5.96; N 8.72; S 9.98; Cl 22.07; found: C 33.48; H 5.79; N 8.77; S 9.70; Cl 22.02[2].

2-[Bis(2-chloroethyl)amino]-2-oxo-4-(sulfidoethane-2-ol)-1,3,2-oxazaphosphorinane (4-(S-ethanol)-sulfidoCP), II. Reaction of 18 mmol crude 4-CHCP with 36 mmol (2.53 ml) 2-mercaptoethanol was achieved. Complete evaporation of the solvent in vacuo under ice-cooling yielded an odorless oily product. Addition of 5 ml CH_2Cl_2 and maintenance of this solution at −20° C for several weeks led to crystallization of II into white needles in druses. In subsequent preparations, when we had crystals available for inoculation, the oily residue crystallized readily when the flask was scratched in the presence of crystals and then kept at −20° C. The crystals were sucked off at room temperature and washed with −20° C cold diethylether to give 5.18 g (85%) II. Recrystallization from CH_2Cl_2/diethylether yielded 4.75 g II (78%), mp 78°–79° C. An appropriate method to obtain II rapidly in the crystalline state and also for high-grade routine purification with minimum loss of the compound was found in its isolation from the oily reaction product by HPLC at room temperature (about 100 mg oil per application). The fractions containing II (k' = 2.86) were collected in an ice/NaCl bath and ice-cooled water was added to decrease the MeOH concentration to 30%. Subsequent freezing (dry ice/acetone) and lyophilization with cooling of the flask to −20° C during the final phase yielded II in the form of an oil[3]. It was dissolved with 1–2 ml CH_2Cl_2 and 3–5 ml diethylether was added. Crystallization started immediately after cooling to 0° C and was completed by portion-wise addition of 20 ml diethylether and exposure to −20° C overnight: ir $\frac{KBr}{max}$ (cm^{-1}): 3260[4] (broad, OH, NH), 2950, 1450, 1230 (P = O), 1125, 1065 (POC), 980, 940, 875, 835, 800, 755 (CCl); ^1H NMR (TMS, DMSO-d_6) δ 1.58–2.39 (m, C_5-H_2, 2 H), 2.39–2.86 (m, SCH_2 + DMSO), 3.06–3.48 (m, 2 NCH_2, 4 H), 3.48–3.80 (m, 2 $NCH_2\underline{CH_2}$ + $SCH_2\underline{CH_2}$, 6 H), 3.80–4.71 (m, C_6-H_2, (C_4-H)/2, 2.5 H), 4.71–4.94 (t, J_{OH,CH_2} = 5.5 Hz, OH + (C_4-H)/2, 1.5 H), 5.41 (d of d, $J_{NH,P}$ = 7.2 Hz, $J_{NH,C4-H}$ = 4.3 Hz, NH, 1 H). On deuterium exchange δ 5.41 disappeared and integration of t δ 4.71–4.94 showed disappearance of one proton to t, (C_4-H)/2, 0.5 H; δ 3.80–4.94 could be divided into δ 4.57–4.94 (d of t, $J_{C4-H,P}$ = 21.5 Hz, $J_{C4-H,C5-H_2}$ = 4.6 Hz, C_4-H, 1 H) and δ 3.80–4.57 (m, C_6-H_2, 2 H). Multiplicity of δ 3.48–3.80 decreased obviously, though overlapped by the HDO signal. Anal. ($C_9H_{19}N_2O_3SPCl_2$) calcd.: C 32.06; H 5.68; N 8.31; S 9.51; Cl 21.03; found: C 31.94; H 5.57; N 8.47; S 9.41; Cl 21.07. Field desorption (FD) MS (m/e): 337 (MH^+, 2 Cl), 275 (2 Cl), 141 ($NH(C_2H_4Cl)_2^+$, 2 Cl).[5]

2-[Bis(2-chloroethyl)amino]-2-oxo-4-sulfido-tert-butyl-1,3,2-oxazaphosphorinane (4-(S-tert-butyl)-sulfidoCP), III. The oily product of the reaction of 0.36 mmol 4-OHCP and 0.72 mmol (81 µl) *tert*-butylmercaptan at −20° C was dissolved in 1 ml diethylether, and about 1 ml petroleum ether 40–60 was added until cloudiness appeared. After the flask had been scratched with a glass rod, the solution was placed on dry ice for several hours and subsequently kept at −20° C for 1–3 days until crystals of III appeared as rectangular colorless platelets. Crystals were sucked off and washed with −20° C cold petroleum ether. The yield was 77 mg (61%), mp 89–90° C, ir $\frac{KBr}{max}$ (cm^{-1}): 3240 (NH), 2960, 1460, 1400, 1370, 1340, 1310, 1270, 1235 (P = O), 1125, 1090, 1045 (POC), 980, 900, 745 (CCl); ^1H NMR (TMS, DMSO-d_6) δ 1.31 (s, $(CH_3)_3$, 9 H), 1.51–2.40 (m, C_5-H_2, 2 H), 3.00–3.80 (m, $2CH_2CH_2Cl$, 8 H), 3.80–4.50 (m, C_6-H_2, 2 H), 4.69 (d of d of t, $J_{C4-H,P}$ = 18.5 Hz, $J_{C4-H,NH} \sim J_{NH,P} \sim$ 4.5 Hz, C_4-H, 1 H), 5.07 (d of d, $J_{NH,P} \sim J_{NH,C4-H} \sim$ 4.5 Hz, NH, 1 H). On deuterium exchange, δ 5.07 disappeared and 4.69 degenerated to d of t, $J_{C4-H,P}$ = 18.5 Hz, $J_{C4-H,C5-H_2}$ = 4.7 Hz. Anal. ($C_{11}H_{23}N_2O_2SPCl_2$) calcd.: C 37.83; H 6.64; N 8.02; S 9.18; found: C 38.01; H 6.39; N 8.13; S 8.91[2].

2-[Bis(2-chloroethyl)amino]-2-oxo-sulfidobenzyl-1,3,2-oxazaphosphorinane (4-(S-benzyl)-sulfidoCP), IV was synthesized from 0.36 mmol 4-OHCP and 0.72 mmol (85 µl) benzylmercaptan and crystal-

2 Electron impact (EI) and field-desorption (FD) mass spectrometric analyses have been published elsewhere [16, 17]

3 The same HPLC procedure was used for purification of 4-OOHCP (k' = 2.28), yielding the compound directly from lyophilization in a crystalline state

4 This value was unaltered when the compound was measured in solution ($CHCl_3$) and after dilution of the sample to the detection minimum

5 Technical data and a detailed FD mass spectrometric study of II will be reported elsewhere

lized as described above for III. The yield was 105 mg (76%) of fine white needles, which were combined into a felt-like voluminous mass, mp 53°–54° C, ir ν_{max}^{KBr} (cm^{-1}): 3165 (NH), 2890, 1490 (aryl H), 1440, 1365, 1305, 1260, 1235 and 1195 (P = O), 1100, 1060 (POC), 990, 975, 950, 895, 775 (CCl), 735, 710; ^1H NMR (TMS, DMSO-d_6) δ 1.58–2.25 (m, C_5-H_2, 2 H), 3.07–3.79 (m, $2CH_2CH_2Cl$, 8 H), 3.84 (s, SCH_2, 2 H), 3.91–4.81 (m, C_6-H_2 + C_4-H, 3 H), 5.50 (d of d, $J_{NH,P} \sim J_{NH,C4-H} \sim 4.4$ Hz, NH, 1 H), 7.31 (s broad, C_6H_5, 5 H). On deuterium exchange δ 5.50 disappeared, 3.91–4.81 degenerated and could be divided into δ 3.91–4.35 (m, C_6-H_2) and δ 4.35–4.81 (d of t, $J_{C4-H,P} = 21.7$ Hz, $J_{C4-H,C5-H_2} = 4.3$ Hz, C_4-H) where the upfield half of d of t was still overlapped by the C_6-H_2 multiplet. Anal. ($C_{14}H_{21}N_2O_2$ S P Cl_2) calcd.: C 43.87; H 5.52; N 7.31; S 8.37; found: C 43.72; H 5.69; N 7.65; S 8.48[2].

Hydrolysis of I–IV

Solutions (1 m*M*) of I–IV in oxygen-free 0.07 *M* phosphate buffer pH 7.0 were kept at 37° C under N_2. At intervals of 1 min aliquots of 900 µl were withdrawn and cooled on ice immediately. Subsequent quantitative determination of either decay of substrate concentration or increase of free thiol concentration for the calculation of half-lives of I–IV was performed by HPLC with solvent MeOH/H_2O (11 : 9 for compound I; 1 : 9 for compound II; 3 : 2 for compound III; and 7 : 3 for compound IV).

Pharmacological Testing

In vitro cytotoxicity[6] of I–IV was tested according to Schmähl and Druckrey [3, 18] by assaying the transplantability of Yoshida ascites tumor cells to rats after incubating the cells for 2 h in increasing concentrations of the test compounds in Ringer buffer. Each cell sample was transplanted IP to a collective of six female Sprague-Dawley rats. A cytotoxic unit (CU) represents the quantitiy of cytotoxic compound that prevents tumor growth in 50% of the animals [3].

Lethal doses (DL_{50})[6] of I and II were estimated by means of single IP injections of the test compounds in physiological saline solution to collectives of three virile Sprague-Dawley rats, mean weight 150 g, or on virile nu/nu mice,[7] II being dissolved in physiological saline solution with $CaCl_2$ [13, 23] equimolar to II. The observation period was 14 days (or 30 days).

Curative doses (DC_{50})[6] were determined by means of single IV injections of I and II dissolved in physiological saline solution to collectives of six female Sprague-Dawley rats 3 h after IP transplantation of Yoshida ascites sarcoma. The observation period was 90 days. For determination of DC_{50} on human mammary carcinoma xenografts (0.15–0.25 g) in female nu/nu mice the test compounds, dissolved in physiological saline solution containing $CaCl_2$ [13, 23] equimolar to II, were injected IV on three occasions at weekly intervals, starting on day 18–25 after transplantation of the xenografts.[7] On day 21 after the first injection the animals were killed and the tumor weights were determined. C represents the tumor weights of the untreated controls, T of treated animals.

6 We are indebted to Prof. N. Brock, Pharmakologische Abteilung of ASTA-Werke, Bielefeld, FRG, for these tests

7 Detailed experimental data will be published elsewhere by G. Voelcker et al. We are indebted to Drs. G. Bastert and H. Fortmeyer, Klinikum der Universität Frankfurt, for providing us with nu/nu mice and xenografts

Results

The 4-(SR)-sulfidocyclophosphamides (I–IV) synthesized according to the procedure described above were obtained in the crystalline state with good yields (Table 1). From the ^1H NMR-coupling constants and IR data (see footnote[a], Table 1) together with the lack of optical activity, we conclude that they are racemic *cis*-isomers (P = O axial/-SR axial). The crystalline compounds, except I, can be stored at room temperature for some months without signs of decomposition. In contrast to this, the noncrystalline diastereomeric forms of I–IV – which have lower R_f values on TLC than I–IV and are produced in smaller amounts – are highly unstable when exposed to temperatures higher than 0° C. As a result of this, even slight contamination of I–IV by their diastereomeric forms leads to significant minor stability, as indicated by yellowing after some hours' exposure to room temperature. Therefore the preparation of I–IV must be accomplished with special attention to stereochemical purity, which was optimally achieved with HPLC as the final purification step.

The data in Table 1 show that our aim of synthesizing stabilized forms of activated CP (4-OHCP) is attained at least by the 4-(S-ethyl)- and the 4-(S-ethanol)-sulficoCP derivatives, with respect to their stability at room temperature, solubility in aqueous solution, and the subsequent spontaneous release of 4-OHCP.

While the first compound mentioned is not very stable at room temperature, the second shows good stability, and also excellent solubility in water (Table 1). Therefore it was favored for further testing. This compound was odourless, in contrast to the others.

4-OOHCP, first synthesized by Takamizawa et al. [20], is nearly as stable as the 4-(SR)-sulfidocyclophosphamides (see Table 1). However, because of the reactive hydroperoxy group, the occurrence of unwanted biological effects in addition to those of activated CP is thought possible [1]. Therefore it cannot strictly be considered as a stabilized form of activated CP.

When dissolved in water, the 4-(SR)-sulfidocyclophosphamides I–IV undergo rapid hydrolysis at physiological pH and temperature and release activated CP (see Fig. 1). The differences in the half-lives of hydrolysis shown in Table 1 reflect both the electron influence of the substituents at the C-4 positions of the oxazaphosphorinane ring (see pK_a values for the corresponding thiols in Table 1) and the sterical effect, demonstrated by the *tert*-butyl derivative (III). If the substituent is a macromolecule, such as bovine serum albumin [24] or a synthetic poly-anion (Hirano et al., submitted for publication), hydrolysis can be almost completely prevented or significantly retarted. Thus, by variation of the sulfido substituent one can obtain stabilized activated CP derivatives with a wide range of hydrolysis rates. Although hydrolysis of the 4-(SR)-sulfidocyclophosphamides leads to an equilibrium that lies decidedly

Table 2. Preliminary evaluation of biological activity of 4-(SR)-sulfidocyclophosphamides

Compounds	Cytotoxicity[a] (CU/μmol)	Toxicity (rats) DL_{50} (mg/kg)	DC_{50}[b] (mg/kg)	DL_{50}/DC_{50} (= TI)
4-(S-ethyl)-sulfidoCP, I	20	100[e]	1.8[e]	55[e]
4-(S-ethanol)-sulfidoCP, II	24	170[e]	3.1[e]	55[e]
4-(S-*tert*-butyl)-sulfidoCP, III	3[c]	n.d.[f]	n.d.	n.d.
4-(S-benzyl)-sulfidoCP, IV	9[c]	n.d.	n.d.	n.d.
4-OHCP	20	150[d]	1.25[d]	120[d]
CP	0	220[d]	1.25[d]	175[d]

[a] On Yoshida ascites cells in vitro
[b] On Yoshida ascites sarcoma-bearing rats, 90-day survivors
[c] Because of low solubility in water (Table 1) these compounds were predissolved in a small volume of oximazone (1-methyl-3-(2-hydroxyethyl)-imidazolidone, ASTA, 1 mg III or IV in 0.5 ml) before Ringer buffer was added
[d] Data from [2]
[e] Approximate values
[f] n.d., not determined

on the side of the products [7], the reaction proceeds to complete breakdown, because the activated CP continously produced undergoes tautomerization through ring opening to the aldophosphamide (AP), which then releases acrolein and the alkylating moiety *N*-lost-phosphorodiamidic-acid (NPDA) by β-elimination [6], as shown in Figure 1 (toxification).

Previously we have found that under in vivo conditions toxification of activated CP can be controlled, depending on the relative concentration of free thiol and activated CP [7, 13]. We suggest that this mechanism may lead to a better understanding of the special mechanism of action of this class of alkylating cytostatics, and especially of the cytotoxic specificity and relatively high cancerotoxic selectivity [10, 4].

The preliminary evaluation of biological and pharmacological activities of the 4-(SR)-sulfidocyclophosphamides I–IV, shown in Table 2, were compared with the corresponding data for activated CP (4-OHCP) and CP, respectively. The data show that 4-(S-ethyl)- and the 4-(S-ethanol)-sulfidoCP exhibit practically the same cytotoxicity to Yoshida sarcoma ascites cells of rats as 4-OHCP, while the 4-(S-benzyl)- and the 4-(S-*tert*-butyl)-sulfidoCP were significantly less active in the transplantation test. Most probably a limited uptake of these derivatives into the cancer cells is a major reason for their minor cytotoxicity.

This in turn would indicate that the intact 4-(SR)-sulfidoCP must enter the cell to exert a cytotoxic effect, and not 4-OHCP produced extracellularly by hydrolysis.

The toxicity after single IV injections to Sprague-Dawley rats and the approximate curative doses of the 4-(S-ethyl)- and the 4-(S-ethanol)-sulfidoCP are also similar to those found for 4-OHCP (Table 2). This indicates that these derivatives also retain the special in vivo characteristic of CP, namely a relatively high TI, which means relatively high cancerostatic selectivity compared with the corresponding value for simple (nonoxazaphosphorinane) nor-*N*-mustard of 2.5 [2].

In thymusless nu/nu mice not only practically identical DL_{50} values were found after single IP injections of 4-(S-ethanol)-sulfidoCP and 4-OHCP (see Table 3), but also very similar cytotoxic effects on xenografts of human mammary carcinoma. This was determined by comparing the increase in tumor weight after three

Table 3. Preliminary evaluation of oncostatic efficacy (human mammary carcinoma xenografts)

Compound	Dose (mg/kg)	Mode of administration	C/T[a]	*n*	DL_{50} (mg/kg)
4-(S-ethanol)-sulfidoCP, II	46	IV	3.7	6	200
4-OHCP	32	IV	2.8	10	150
CP	100	IP	8.3	10	500

[a] C/T, mean tumor weights of untreated/treated xenografts; dose: 20% of DL_{50} (see "Materials and Methods")

weekly IV injections of equitoxic doses (20% of the DL_{50}) against the increase in the controls.

Equitoxic doses of CP show a higher cytostatic effect against both Yoshida sarcoma of the rat (Table 2) and human mammary carcinoma xenografts to nu/nu mice (Table 3). Probably this is the result of different pharmacokinetics of the activated CP (4-OHCP) after injection of CP compared with the injection of 4-(SR)-sulfidoCP or of activated CP itself. Current studies on the influence of the pharmacokinetics of activated CP and the toxic and curative effect seem to support this assumption.

Discussion

Latent cytotoxic drugs, such as CP and analogous compounds of the *N*-(2-chloroethyl)-amido-oxazaphosphorinane series, require enzymatic activation in the liver to exert their cytotoxic action in vivo (see Fig. 1). Therefore the activated metabolites that are also available by synthesis [20, 15, 21, 11] should theoretically be the best substitutes for the parent drugs, since they possess all the wanted pharmacological properties of the precursor, especially a high cytotoxic specificity, as we recently demonstrated for activated CP (4-OHCP) [4]. Unfortunately, high instability of the activated derivatives in general excludes their use in the clinical application.

The aim of the present study was to synthesize stable derivatives of activated *N*-(2-chloroethyl)-amido-oxazaphosphorinanes, which at physiological pH and temperature, without enzymatic transformation, release the activated form. As we show in this paper, 4-(SR)-sulfido derivatives of CP can fulfill these requirements. In biological assays two of the 4-(SR)-sulfidoCP derivatives synthesized were shown to exhibit cytotoxic activities resembling those of activated CP. These compounds may therefore provide new therapeutic possibilities for local chemotherapy, such as intrapleural or intraperitoneal instillation and organ perfusion, for which CP and analogous latent drugs are ineffective. Furthermore, for systemic chemotherapy an optimalization of the pharmacokinetics of the activated metabolite of CP seems to be possible by way of a controlled application of 4-(SR)-sulfidoCP derivatives that bypass the limits of enzymatic activation of these drugs in the liver.

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