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Lipophilic disulfide prodrugs – syntheses and disulfide bond cleavage

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Summary

Various drugs bearing a thiol or thione function were coupled with activated lipophilic thiol derivatives to afford unsymmetrical disulfides. Synthetic methods were developed to introduce one or two long alkyl chains into drug molecules in order to obtain highly lipophilic prodrugs. These might be suitable to form bilayers or for integration into liposomes. Disulfide bond cleavage was assessed by preincubation of the 6-MP prodrug **2** in serum followed by a bioassay. The preincubation period did not increase the inhibitory potency of the prodrug on lymphocyte proliferation as compared to the parent drug. Thus disulfide bond cleavage of prodrug **2** is assumed to be an active cellular process.

Introduction

A variety of drugs with different applications contain nucleophilic thio-functions often considered to be essential for a pharmacological effect, because of their high reactivity (Jocecyn, 1972).

Prodrugs of such compounds are usually prepared by acylating or alkylating the thio-function. In contrast, the reaction with appropriate thiol derivatives which yields unsymmetrical disulfides is rarely performed (Higuchi, 1987). Lipophilic prodrugs of hydrophilic drugs are of particular interest since they show improved absorption, intracellular uptake, and passage of the blood–brain barrier. Prevention of fast biodegradation and sustained release effects are further advantages of drug lipophilisation (e.g. Matsushita et al., 1981; Martin et al., 1987; Hong et al., 1988; Waranis and Sloan, 1988).

In cancer chemotherapy and some other areas of research good results were obtained with the so-called 'integrated prodrug approach': drugs are covalently coupled to long alkyl chains or steroid moieties (chemical modification). In contrast to unmodified drugs which are often poorly entrapped into liposomes, the lipophilic prodrugs can be integrated with high rates and high stability into drug delivery systems such as liposomes or microspheres (physical modification) (e.g. Mizushima et al., 1983; Sasaki et al., 1985, 1986, 1987; Schwendener et al., 1985; Hashida et al., 1988).

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In the present study, we synthesized various highly lipophilic disulfide prodrugs of 6-mercaptopurine, D-penicillamine, thiamine, 6-methyl- and 6-propylthiouracil. To investigate the consequences of this chemical modification on liberation of the parent drug we used 6-MP prodrug **2** as a model compound. The biodegradation of this prodrug can be monitored by evaluation of the antiproliferative potency on mitogen-induced human peripheral blood lymphocytes. The stability of prodrug **2** in thiol-containing serum was tested to determine the extent and localisation of the disulfide bond cleavage reaction in vitro.

Materials and Methods

Synthesis

Melting points were determined in capillary tubes using a Büchi 510 melting point apparatus and were not corrected. IR (KBr) spectra were recorded on a Perkin–Elmer FT-IR spectrometer 1750. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker WP-80 spectrometer (80 MHz) with tetramethylsilane as internal standard. UV spectra were recorded on a Beckman DU-50 spectrophotometer. Elemental analyses and FD-MS spectroscopy were performed by the Chemical Institute, University of Tübingen. Silica gel 60 (Merck), 35–70 mesh, was used for column chromatography. Thin-layer chromatography was performed on silica gel 60 F_{254} plates (Merck). The spots were detected by UV or by spraying with neutral $KMnO_4$ solution.

Chemicals were commercially available or prepared as described below.

Thiophthalimides (compounds **1** and **9**) and thiosulfinate (compound **5**) were prepared as described (Müller and Roth, 1989).

Compound 6: S-[1,2-bis-(octadecyloxycarbonyl)ethyl]-1,2-bis-(octadecyloxycarbonyl)ethanethiosulfinate

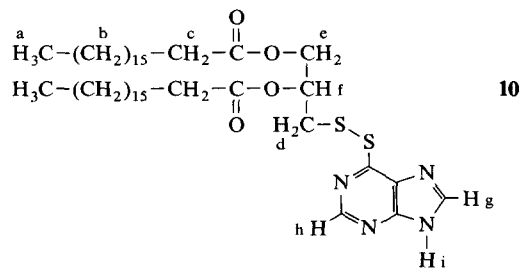
This compound was prepared in the same way as thiosulfinate (**5**) by oxidation of bis(1,2-dioctadecyloxycarbonylethyl)disulfide (Müller and Roth, 1989) in dichlormethane by use of *m*-chloroperbenzoic acid, and was used without further purification.

Compound 2: 6-(octadecyldithio)purine

4.32 g (10.0 mmol) **1** and 1.70 g (10.0 mmol) 6-mercaptopurine monohydrate were refluxed for 3 h in 50 ml ethanol. After cooling to room temperature, 200 ml of hot water were added. The precipitated product was collected by filtration and washed several times with hot water to remove the by-product phthalimide. After drying, the crude product was dissolved in chloroform and precipitated with petrolether. Yield: 3.40 g (78%). White crystalline substance, soluble in chloroform/ethanol (10 : 1), slightly soluble or in-

TABLE 1

^{1}H-NMR data for 6-mercaptopurine derivatives



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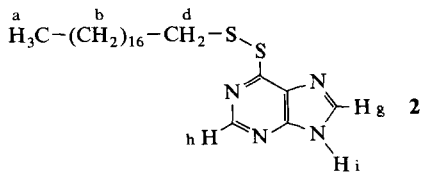


2

Assignment	**10** [a]	**2** [b]	**2** [c]
a	0.88, t, 6H	0.87, t, 3H	0.87, t, 3H
b	1.25, br s, 60H 1.56, m	1.25, br s 32H 1.55, m	1.29, m 32H
c	2.31, q, 4H		
d	3.24, d, 2H	2.93, t [d]	3.13, t, 2H
e	4.39, t, 2H		
f	5.38, m, 1H		
g	8.25, s, 1H	8.19, s, 1H	[d]
h	8.88, s, 1H	8.80, s, 1H	9.18, s, 1H
i	12.42, br s, 1H	10.94, br s, 1H	

[a] Chloroform-d_1; [b] Chloroform-d_1 : dimethylsulfoxide-d_6 = 5 : 1; [c] Pyridine-d_5; [d] solvent overlapping.

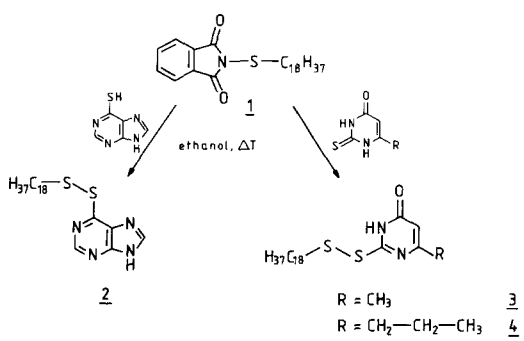
soluble in other solvents. m.p.: 122 °C. $C_{23}H_{39}N_4S_2$ (435.7). Calc.: C 63.40, H 9.02, N 12.85, S 14.71; found: C 63.49, H 9.18, N 12.59, S 14.64. FT-IR: ν [cm^{-1}]: 3436 (NH); 2918, 2850 (CH_2); 1593, 1569 (aromat.). ^{1}H-NMR: see Table 1. FD-MS: m/e = 437 (100%). UV_{max} (dichlormethane/ethanol, 9 : 1) = 282 nm (ϵ = 8949).

Compound 3: 2-(octadecyldithio)-6-methyl-3H-pyrimidine-4-one

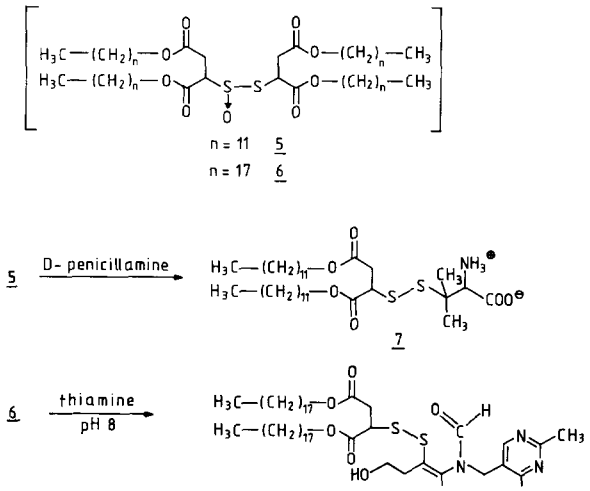
4.32 g (10.0 mmol) **1** and 1.42 g (10.0 mmol) 6-methyl-2-thiouracil were refluxed in ethanol for 6 h. The precipitation of the product was accomplished upon addition of water. After filtration it was washed several times with hot water. Yield: 2.39 g (56%). White crystals, soluble in chloroform. m.p.: 80 °C. $C_{23}H_{42}N_2O_1S_2$ (426.7). Calc.: C 64.73, H 9.92, N 6.56, S 15.52; found: C 64.82, H 10.06, N 6.20, S 15.27. FT-IR: ν [cm^{-1}]: 3432 (NH); 2919, 2850 (CH_2); 1672, 1644 (C=N). ^{1}H-NMR (chloroform-d_1): δ [ppm]: 0.88 (t; 3H; CH_3); 1.26, 1.53 (br s + m; 32H; CH_2 and CH_2–C<u>H</u>$_2$–S); 2.27 (s; 3H; C_6–CH_3); 2.83 (t; 2H; CH_2–S); 6.07 (s; 1H; C_5–H).

Compound 4: 2-(octadecyldithio)-6-propyl-3H-pyrimidine-4-one

Preparation was accomplished in analogy to **3** from 4.32 g (10.0 mmol) **1** and 1.70 g (10.0 mmol) 6-propyl-2-thiouracil. Yield: 1.77 g (39%). White crystalline substance, soluble in chloroform. m.p.: 62 °C. $C_{25}H_{46}N_2O_1S_2$ (454.8). Calc.: C 66.02, H 10.19, N 6.15, S 14.09; found: C 66.01, H 9.82, N 6.54, S 13.92. FT-IR: similar to compound **3**.



Scheme 1.

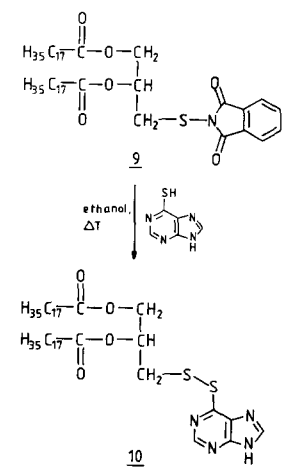


Scheme 2.

^{1}H-NMR (chloroform-d_1): δ [ppm]: 0.96 (t; 6H; CH_3); 1.26, 1.54 (br s + m; 34H; CH_2, CH_2–CH_2–S and propyl-CH_3–CH_2); 2.48 (t; 2H; propyl-C_2H_5–CH_2); 2.83 (t; 2H; CH_2–S); 6.06 (s; 1H; C_5–H); 7.83 (d; 1H; N_3–H).

Compound 7: S-[1,2-bis-(dodecyloxycarbonyl)ethylthio]penicillamine

Diluted HCl solution was added to a suspension of 1.35 g (9.0 mmol) D-penicillamine in 10 ml methanol until a clear solution was obtained. This solution was slowly added to a dichlormethane



Scheme 3.

solution of **5**, prepared from 4.86 g (5.0 mmol) bis-(1,2-didodecyloxycarbonylethyl)disulfide. The product was separated upon addition of methanol and purified by column chromatography. Lipophilic by-products were eluted with dichlormethane followed by elution of the product with dichlormethane/methanol (3:1). Yield: 3.05 g (48%). Yellowish glutinous compound, soluble in chloroform. $C_{33}H_{63}N_1O_6S_2$ (634.0). Calc.: C 62.51, H 10.01, N 2.20, S 10.11; found: C 62.97, H 10.06, N 1.82, S 10.38. FT-IR: ν [cm^{-1}]: 3431 (NH_3^+); 2925, 2854 (CH_2); 1737 (C=O, ester); 1635 (NH_3^+). ^{1}H-NMR (chloroform-d_1): δ [ppm]: 0.88 (t; 6H; CH_3); 1.26, 1.55 (br s + m; 46H; $CH_2 + 2 \times CH_3$); 2.87 (m; 2H; bb-CH_2); 4.07–4.14 (m; 6H; CH_2OOC + bb-CH + D-pen-CH); (bb = backbone, D-pen = D-penicillamine).

*Compound **8**: 2-[2'-methyl-4'-aminopyrimidyl-(5')]-methylformamino-5-hydroxy-Δ2-pentenyl-(3)-[1,2-bis-(octadecyloxycarbonyl)ethyl]disulfide*

3.37 g (10.0 mmol) thiamine · 2 HCl were dissolved in a small amount of water. A pH value of 8.0 was adjusted by addition of 1 N NaOH solution. 10 ml ethanol were added and the mixture was slowly added to a dichlormethane solution of **6** prepared of 6.54 g (5 mmol) bis-(1,2-dioctadecyloxycarbonylethyl)disulfide. The mixture was cautiously evaporated to dryness. The product was purified by column chromatography with dichlormethane/ethanol (9:1). Yield: 2.34 g (25%). Slightly yellow crystals, soluble in chloroform. R_F (chloroform) = 0.4. m.p.: 45 °C. $C_{52}H_{94}N_4O_6S_2$ (935.5). Calc.: C 66.76, H 10.12, N 5.98, S 6.85; found: C 66.69, H 10.39, N 4.07, S 6.64. FT-IR: ν [cm^{-1}]: 3435 (OH, NH); 2918, 2851 (CH_2); 1735 (C=O, ester); 1657 (C=C, C=N); 1599 (NH_2). ^{1}H-NMR (chloroform-d_1): δ [ppm]: 0.88 (t; 6H; CH_3); 1.26, 1.57 (br s + m; 64H; CH_2); 2.48 (s; 3H; pyrimidine-CH_3); 2.62 (s; 3H; CH_3–C=C); 2.70–3.03 (m; 4H; bb-CH_2 and -CH_2–CH_2–OH); 3.65–3.85 (m; 4H; -CH_2-OH; OH and bb-CH); 4.08 (t; 4H; CH_2OOC); 5.16 (s; 2H; N–CH_2-pyrimidine); 7.09 (s; 1H; pyrimidine-C_6–H); 8.12 (s; 2H; NH_2); 8.60 (s; 1H; CHO). UV_{max} (diethylether/dichlormethane, 8:2): 236 nm (ϵ = 4868); shoulder at 266 nm (ϵ = 3096).

*Compound **10**: 6-[2,3-bis-(octadecanoyloxy)propyl-1-dithio]purine*

1.97 g (2.5 mmol) **9** and 0.43 g (2.5 mmol) 6-mercaptopurine · $1H_2O$ were refluxed in 20 ml ethanol for 5 h. After cooling to room temperature, 10 ml of water were added and the precipitated product was isolated by filtration. Purification was performed by column chromatography with dichlormethane/ethanol (9:1). Yield: 1.33 g (67%). White crystalline compound, soluble in chloroform. m.p.: 83 °C. $C_{44}H_{78}N_4O_4S_2$ (791.3). Calc.: C 66.79, H 9.93, N 7.08, S 8.10; found: C 66.71, H 9.71, N 6.95, S 8.06. FT-IR: ν [cm^{-1}]: 3436 (NH); 2919, 2851 (CH_2); 1740 (C=O, ester); 1568 (C=C, aromat.). ^{1}H-NMR: see Table 1. ^{13}C-NMR (chloroform-d_1): δ [ppm]: 14.1 (CH_3); 22.7 (CH_3–CH_2); 24.9 (CH_2–CH_2–CO); 29.2, 29.4, 29.5, 29.7 (CH_3–CH_2–CH_2–[CH_2]$_{12}$); 31.9 (CH_3–CH_2–CH_2); 34.1 (CH_2–COO–CH_2); 34.3 (CH_2–COO–CH); 39.9 (CH_2–S); 63.5 (O–CH_2–CH); 70.0 (O–CH); 130.6 (C_5); 142.0 (C_8); 150.3 (C_4); 152.2 (C_2); 159.2 (C_6). FD-MS: m/e = 791 (100%). UV_{max} (dichlormethane/ethanol, 9:1): 282 nm (ϵ = 7468).

Pharmacological test

Phytohemagglutinin was obtained from Wellcome, fetal calf serum from Seromed, Berlin, RPMI 1640 and glutamine from Flow, ^{3}H-TdR from Amersham-Buchler, Braunschweig, Hank's BSS from Gibco-BRL and Ficoll–Hypaque from Pharmacia.

Phytohemagglutinin-induced lymphocyte proliferation test

Human peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood and were separated by Ficoll density gradient centrifugation (150 × *g*, 30 min), washed with Hank's BSS three times (150 × *g*, 10 min) and resuspended in RPMI 1640 (supplemented with glutamine). Cell viability was determined by acridine orange staining. 10^5 cells were incubated in 200 µl RPMI 1640 supplemented with glutamine and 10% fetal calf serum in flat bottom microtiter plates. The cells were stimulated with

phytohemagglutinin (0.5 μg/ml) and incubated for 40 or 72 h, respectively, at 37 °C (in a humidified 5% CO_2 atmosphere). 24 h before cell harvesting, the cells were pulsed with ^{3}H-TdR (1 μCi). After incubation, the cells were harvested on filter papers, and dried. Cell-bound radioactivity was determined by liquid scintillation counting and ranged from 30 000 to 100 000 cpm for the control cultures. Incorporated radioactivity is proportional to cell proliferation. Lymphocyte proliferation was performed n times and statistical significance was calculated by analysis of variance. (6-MP: $n = 28$; azathioprine: $n = 8$; prodrug **2**: $n = 22$; preincubation experiment: $n = 1$ in hexaplicates.) Prodrug **2** was solubilized by sonication and intermittent cooling in RPMI 1640 supplemented with 10% fetal calf serum until the solution was clear.

Results

Chemistry

Prodrugs **2**, **3** and **4** of the thyreostatics methylthiouracil (MTU) and propylthiouracil (PTU) and of the cytostatic and immunosuppressive agent 6-mercaptopurine (6-MP) with *one* alkyl chain were obtained by reaction of the drugs with *N*-octadecylthiophthalimide (**1**). These thio-compounds exist partly in the highly nucleophilic zwitterionic forms. Compound **1** reacts as an electrophilic thiol derivative with the thiouracils and the thiopurine.

Derivatisation of drugs with *two* long alkyl chains is expected to result in increased lipophilicity. Such prodrugs are similar to natural bilayer forming compounds like phospholipids. They are better incorporated into liposomes or may spontaneously form vesicles.

To obtain double-chain prodrugs, the thiosulfinates **5** and **6** are reacted with D-penicillamine or thiamine, respectively, to yield prodrugs **7** and **8**. Some lipophilic prodrugs of thiamine have gained broad application because of their improved pharmacokinetic properties (Dannhardt and Eger, 1985). In alkaline solution, the thiazolidine ring of thiamine is opened. This 'thiol form' can be used for the preparation of mixed disulfides.

Heterocyclic thiones like 6-MP do not react with thiosulfinates such as **5** and **6**. Therefore diacylglycerothiophthalimide **9** was selected as a lipophilic thiol derivative to synthesize 6-MP prodrug with two alkyl chains. This results in high yields of prodrug **10**.

The structures of the novel disulfide prodrugs were confirmed by IR-, NMR- and partially by UV-data. Since the 6-MP prodrug **2** shows only low solubility in commonly used solvents for NMR spectroscopy the ^{1}H-NMR spectra were recorded in two solvent systems. This confirmed the proposed structure of prodrug **2** (Table 1).

Disulfide cleavage

Prodrugs must be converted to their parent drugs to exert a pharmacological effect. Ideally, the release of the drug should occur at the target site. Disulfides can be cleaved in vivo by enzymatic reduction or non-enzymatically by thiol-disulfide exchange. Although disulfide bond cleavage is an important physiological process only few studies concerning the cleavage mechanism under physiological conditions have been published (Shen et al., 1985).

We tested the 6-MP prodrug **2** as a model compound. To evaluate whether the active drug is released, we tested the antiproliferative potency in a bioassay based on the inhibition of lymphocyte proliferation. 6-MP prodrug was demonstrated to inhibit mitogen-induced proliferation of human peripheral blood lymphocytes. Figure 1 shows a typical dose–effect curve for 6-MP, prodrug **2**, and prodrug azathioprine. Azathioprine is a thioether prodrug of 6-MP with prolonged action. In vivo, it is quantitatively metabolized to 6-MP and an imidazole derivative within hours. The imidazole derivative is supposed to exert an additional antiproliferative activity (Sauer et al., 1988). In this test system 6-MP exhibits the strongest effect, while azathioprine and **2** have to be degraded prior to their inhibition of lymphocyte proliferation. **2** is least effective, which indicates that its metabolism to 6-MP is much slower compared to azathioprine. The latter is degraded by nucleophils such as thiols and amines in serum.

To investigate the extent of disulfide bond cleavage in serum supplemented culture medium

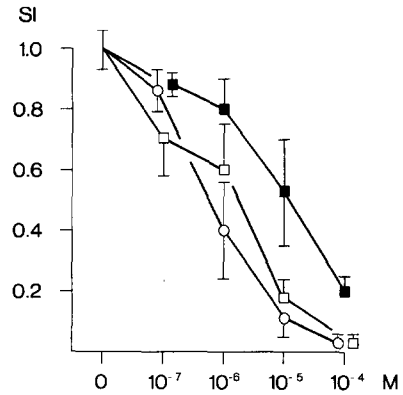


Fig. 1. Suppression of PHA-induced proliferation of human lymphocytes by 6-MP (white circles), azathioprine (white squares), and prodrug **2** (black squares). Proliferation is expressed as stimulation index (SI) relative to control cultures without drug (SI = 1).

prodrug **2** and 6-MP were preincubated in serum-containing, cell-free culture medium at 37 °C for 24, 48, 72 and 96 h. Preincubation was followed by evaluation of antiproliferative activity in a short-time lymphocyte proliferation test over 40 h. Preincubation in serum-supplemented culture medium did not enhance the antiproliferative potency of **2** (see Fig. 2). If **2** was already degraded in the medium, the dose–effect curve of **2** should approach that of 6-MP. We therefore concluded that in this assay system the disulfide bridge of **2** is not cleaved in serum containing medium to a measurable degree. Since prodrug **2** inhibits lymphocyte proliferation, disulfide bond cleavage is assumed to be an active cell-dependent process.

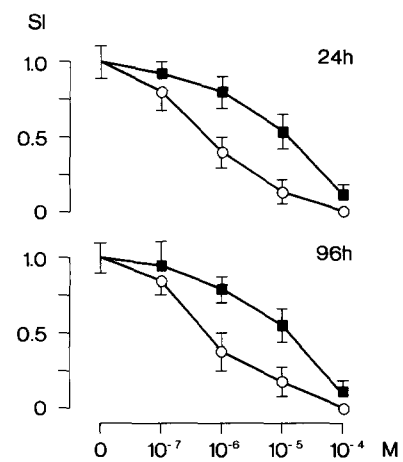


Fig. 2. Effect of preincubation of 6-MP (white circles) and prodrug **2** (black squares) in cell-free serum-supplemented culture medium prior to lymphocyte stimulation. After preincubation at 37 °C for 24–96 h, human PBMC were added and stimulated by PHA for 40 h. Proliferation is expressed as stimulation index (SI).

Incorporation into unilamellar soybean lecithin liposomes significantly improved the immunosuppressive and antiproliferative potency of prodrug **2** (to be published elsewhere).

Conclusion

Coupling of thio-drugs to lipophilic compounds via disulfide bridging is an efficient and feasible method to prepare lipophilic prodrugs. Various synthetic pathways were evaluated to obtain highly lipophilic prodrugs in reasonable yields. Broad applicability is demonstrated by synthesizing prodrugs of various thiol and thione drugs different in structure and pharmacodynamics. One of the synthesized prodrugs, a prodrug of 6-MP, was demonstrated to exert an antiproliferative effect on mitogen-induced human lymphocytes. We therefore concluded that the parent drug is liberated by disulfide bond cleavage. This cleavage reaction was shown to depend on the presence of cells.

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