Synthesis, Cytotoxicity, and Antitumor Activity of Copper(II) and Iron(II) Complexes of ^{4}*N*-Azabicyclo[3.2.2]nonane Thiosemicarbazones Derived from Acyl Diazines

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A series of thiosemicarbazones (TSCs) (bearing a ^{4}N-azabicyclo[3.2.2]nonane moiety) derived from 3-acylpyridazines, 4-acetylpyrimidines, and 2-acetylpyrazines (**1**−**8**) were synthesized as potential antitumor agents. TSCs **1**−**8** exhibited potent cytotoxic activity against human acute lymphoblastic leukemia CCRF-CEM cells (IC_{50} = 0.05−0.77 μM) and colon adenocarcinoma HT-29 cells (IC_{50} = 0.011−2.22 μM). Copper II complexes of TSCs **1**−**8** showed significant improvement in cytotoxic activity against HT-29 cells (IC_{50} = 0.004−1.51 μM) by a factor of 3. However, complexation of ligands **1**, **2**, **4**, and **6** with Fe(II) results in lowering of cytotoxic activity by a factor of ∼7. In clonogenic assays involving human tumor cells of different tumor origins, compounds **5**, **7**, **8**, and their copper complexes **5Cu(II)**, **7Cu(II)**, and **8Cu(II)** exhibited remarkable cytotoxic activities with mean IC_{50} values of 6, 0.18, 1, 1, 0.37, and 0.37 nM, respectively. In particular, the compounds were highly effective against human colon carcinoma and large and small cell lung carcinoma cells. The TSC derivative **5** was evaluated in vivo in nude mice bearing LXFL 529 human large cell lung carcinoma cells. With respect to antitumor activity, application of 30 mg/kg/d resulted in moderate inhibition (42%) of tumor growth. No effect on tumor growth was observed at a dose of 10 mg/kg/d. However, a dose of 40 or 60 mg/kg/d resulted in 50 and 75% death, respectively, in the treated mice, indicating the high toxicity of these compounds. Using human liver microsomes, compound **5** was found to be rapidly and highly metabolized in vitro. In actual fact, only 2% of the unmetabolized compound could be detected in the incubation medium after 5 min. The IC_{50} for cell proliferation (0.006−0.022 μM) elicited by these compounds is much lower than that of the inhibition of [^{14}C]cytidine incorporation into DNA (0.18−3.32 μM). These compounds are also noncell cycle specific agents. Interestingly, compounds **5**, **5Cu(II)**, and **8** were found to be potent inducers of apoptosis in Burkitt's lymphoma cells.

Introduction

The antitumor agent hydroxyurea (HU) is a clinically useful drug for the treatment of a wide range of solid tumors as well as acute and chronic leukemia.[1] HU specifically inhibits DNA synthesis, and the primary site of action is the highly regulated enzyme ribonucleotide reductase (RR).[2] RR reduces ribonucleotides to provide deoxyribonucleotides which are the building blocks for the synthesis of DNA. This reaction is the rate-limiting step for DNA synthesis, and therefore the enzyme plays an important role in the regulation of cell division.[3] The mammalian RR consists of two nonidentical protein subunits commonly referred to as M1 and M2. Protein M1 is a dimer and contains substrate and allosteric binding sites. Protein M2, also a dimer, contains stoichiometric amounts of non-heme iron and a unique tyrosyl free radical that is essential for reductase activity.[4,5] HU inhibits RR by interacting with the M2 subunit to inactivate the tyrosyl free radical.[6] However, considering the fact that HU is a relatively poor inhibitor of RR and has a short serum half-life,[7] other potent inhibitors of this enzyme may be useful antitumor agents.

One such agent described in the literature is 2-formylpyridine thiosemicarbazone. It was found to possess moderate antileukemic activity in mice bearing leukemic cells.[8] Following this initial report, a large series of TSCs derived from 2-formylpyridines,[9] isoquinoline-1-carboxaldehydes,[9] and 2-acetylpyridine[10] were synthesized and evaluated for antitumor activity against a wide spectrum of transplanted murine neoplasms. From these studies, 5-hydroxypyridine TSC was chosen for clinical phase I evaluation. However, it showed extremely weak antileukemic activity that was attributed to its short half-life in man. It was additionally accompanied by high in vivo toxicity.[11] Moreover, these compounds exhibit poor water solubility.[12] The formation of complexes with transition metals has also been

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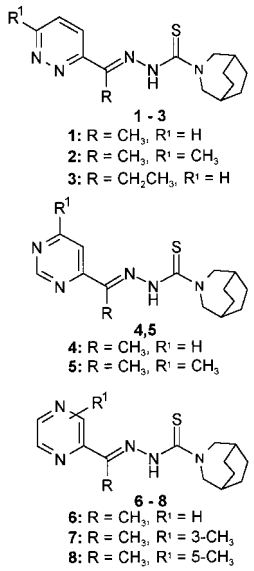


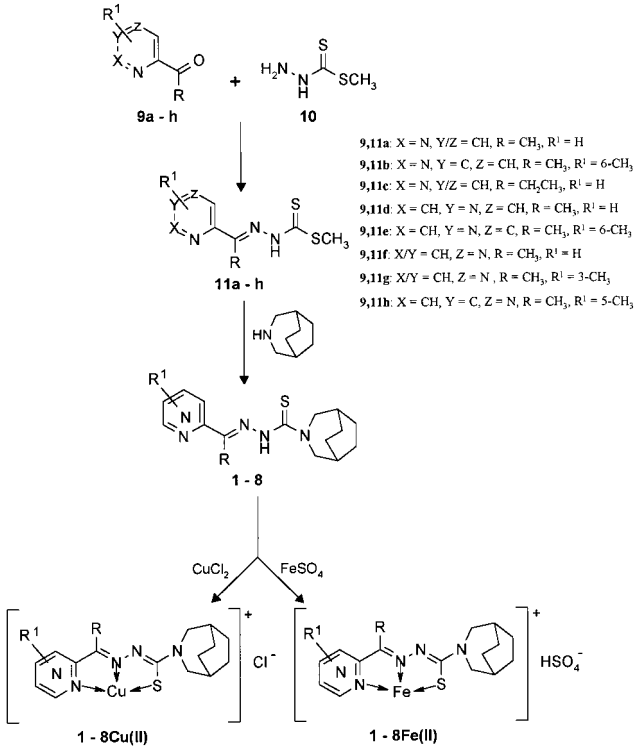
Figure 1.

implicated in the mechanism of action of α-(*N*)-heteroaromatic TSCs. It has been shown that complexation of these TSCs, especially with iron and copper, result in compounds that are potent cytotoxic agents as well as inhibitors of DNA synthesis as compared to the uncomplexed ligand.[10,13] The iron chelate of 1-formylisoquinoline TSC has been shown to inhibit RR activity in a manner similar to HU by destroying the tyrosyl free radical of the protein M2 subunit.[14,15]

For the above reasons, there is continued interest in finding TSC derivatives that may circumvent some of these unfavorable properties. One way we hoped to achieve this was by replacing the 2-formyl- or 2-acetylpyridine substructure with the diazine isostere. Thus, we synthesized a large series of TSCs derived from formyl and acetyldiazines and evaluated their antiherpetic and anti-HIV activity in vitro.[16,17] Although none of the compounds studied showed any significant antiviral activity, conclusions that could be drawn were (i) the replacement of the 2-pyridyl moiety by a diazinyl function, especially the 1,2-diazine, results in significant improvement in water solubility (factor ∼100);[16] (ii) the diazinyl-derived TSCs turned out to be less cytotoxic as compared to the pyridyl congeners (factor ∼ 50), and (iii) moderate synergism could be detected for the relatively noncytotoxic diazinyl-derived TSCs with the antiviral drugs ACV (antiherpes) and AZT (anti-HIV).[17] Also, clear structure−activity relationships could be established concerning the cytotoxic activity of the compounds. Replacement of the terminal primary or secondary amino function by a tertiary amino group results in a drastic enhancement of cytotoxic activity.[17]

In view of these findings, we considered it of high interest to study the effects of acyldiazinyl TSCs bearing an N^4-azabicyclo[3.2.2]nonane group on human tumor cells in vitro and in vivo. In this paper, we describe the synthesis and the results of the antiproliferative and antitumor activities of compounds **1**−**8** (Figure 1), including their copper(II) and iron(II) complexes. In addition, the effects of selected compounds on (i) incorporation of [^{14}C]cytidine into DNA, (ii) the cell cycle, and (iii) the induction of apoptosis in Burkitt's lymphoma cells were also examined.

Scheme 1



Chemistry

The novel thiosemicarbazone derivatives **5**, **7**, and **8** were obtained in satisfactory yields by reacting the methyl hydrazinecarbodithioates **11e**, **g**, and **h** with 3-azabicyclo[3.2.2]nonane in refluxing methanol (Scheme 1). Compounds **11e**, **g**, and **h** were easily accessible by the condensation of ketones **9e**, **g**, and **h** with methyl hydrazinecarbodithioate (**10**) in 2-propanol at 65−70 °C. The thiosemicarbazone derivatives **1**−**4** and **6**, as well as the methyl hydrazinocarbodithioates **11a**−**d** and **f** have been reported earlier.[16,17] For the synthesis of ketones **9a**−**h** see refs 16−19. Previously, we have shown that the TSC derivatives **1**−**4** and **6** were *E*-configurated isomers.[17] This was determined by using ^{1}H NMR spectroscopy and homonuclear NOE-difference experiments. The most characteristic difference in the chemical shifts of the *E*- and *Z*-isomeric forms are in the *E*-form δ(NH) = 9−12 ppm and in the *Z*-form δ-(NH) = 14−15 ppm.[20] Thus, considering these findings, *E*-configuration is assigned to compounds **5**, **7**, and **8** of which δ(NH) ∼9.68−10.32 ppm.

The products of the chloro-Cu(II) complexes **1**−**8Cu-(II)** were obtained by heating one equivalent each of the free ligands **1**−**8** and $CuCl_2$ in dry ethanolic solutions at 40 °C. For the products of the sulfate-Fe(II) complexes **1**, **2**, **4,** and **6Fe(II)**, equimolar amounts of the ligands **1**, **2**, **4**, and **6** and ferrous sulfate heptahydrate were mixed at room temperature in dry ethanol and stirred further at this temperature overnight. On the basis of the microanalytical data obtained, it can be concluded that the Cu(II) and Fe(II) complexes obtained exist in a mono-aligned fashion that requires a ratio of 1:1:1 for ligand, metal ion, and counterion (i.e., Cl^- or HSO_4^-). All new compounds were purified by crystallization from appropriate solvents, and the melting points as well as elemental analyses are given in

Table 1. Physical Data and Inhibition of Cell Proliferation of Thiosemicarbazones **1**−**8**, Copper Complexes **1**−**8Cu(II)**, and Iron Complexes **1**, **2**, **4**, and **6Fe(II)** Thereof[a]

compd	yield %	mp °C (solvent)[b]	formula	cytotoxic effect IC_{50} (μM) CCRF-CEM	HT-29
1	ref 16			0.33	2.22
1Cu(II)	67	229−232 (ACN/MeOH)	$C_{15}H_{20}ClCuN_5S$	0.30	1.51
1Fe(II)	75	>300 (MeOH)	$C_{15}H_{20}FeN_5S\cdot HSO_4$	0.82	0.75
2	ref 17			0.60	0.49
2Cu(II)	65	255−257 (EtOH)	$C_{16}H_{22}ClCuN_5S\cdot 0.5H_2O$	0.13	0.87
2Fe(II)	90	>300 (MeOH)	$C_{16}H_{22}FeN_5S\cdot HSO_4\cdot 1.5H_2O$	0.81	0.77
3	ref 16			0.77	1.37
3Cu(II)	79	>300 (DMF/DIPE)	$C_{16}H_{22}ClCuN_5S$	1.16	0.40
4	ref 17			0.02	0.05
4Cu(II)	92	267−269 (DMF)	$C_{15}H_{20}ClCuN_5S$	<0.01	<0.01
4Fe(II)	93	>300 (DMF/EtOH)	$C_{15}H_{20}FeN_5S\cdot HSO_4$	0.07	0.06
5	98	165−167 (EtOH)	$C_{16}H_{23}N_5S$	0.018	0.038
5Cu(II)	85	253−256 (DMF/DIPE)	$C_{16}H_{22}ClCuN_5S$	0.010	0.004
6	ref 17			0.011	0.011
6Cu(II)	94	>300 (DMF/EtOH)	$C_{15}H_{20}ClCuN_5S$	<0.01	<0.01
6Fe(II)	95	297−300 (DMF/EtOH)	$C_{15}H_{20}FeN_5S\cdot HSO_4\cdot H_2O$	0.07	0.07
7	94	122−124 (EtOAc)	$C_{16}H_{23}N_5S$	0.005	0.023
7Cu(II)	79	260−262 (DMF/DIPE)	$C_{16}H_{22}ClCuN_5S$	0.006	0.028
8	90	142−145 (EtOH)	$C_{16}H_{23}N_5S$	0.011	0.035
8Cu(II)	85	278−280 (DMF/MeOH)	$C_{16}H_{22}ClCuN_5S$	0.005	0.004

[a] Cell proliferation was quantitated as described in the Experimental Section. IC_{50} was determined with CalcuSyn software from Biosoft, Cambridge, UK. Data represent the means (SD ± 0.009−0.242) of at least three independent experiments in which duplicate determinations were taken within each experiment. [b] ACN = acetonitrile, DIPE = diisopropyl ether, DMF = dimethylformamide, EtOAc = ethyl acetate, EtOH = ethanol, MeOH = methanol.

Table 1. The structures of compounds **5**, **7**, **8**, **11e**, **11g**, and **11h** were confirmed by IR and NMR spectroscopy.

Biological Results and Discussion

In Vitro Cytotoxic Studies. All substances were evaluated for cytotoxic activity in vitro using human acute lymphoblastic leukemia (CCRF-CEM) and colon adenocarcinoma (HT-29) cell lines. The data in micromolar are shown in Table 1 as IC_{50} values. The free ligands **1**−**8** exhibited potent cytotoxic activity against CCRF-CEM cells with IC_{50} of 0.005−0.77 μM and HT-29 cells with IC_{50} of 0.011−2.22 μM. Considering the parent heterocycles, the 4-acetyl pyrimidine and the 2-acetyl pyrazine derived TSCs **4**, **5**, and **6**−**8** turned out to be highly potent cytotoxic agents (IC_{50} = 0.005−0.05 μM) as compared to the 3-acyl pyridazine derived TSCs **1**−**3** (IC_{50} = 0.33−2.22 μM) by a factor of 44 to 66 depending on the cell line. Complexation of the ligands with copper II results in compounds (**1**−**8Cu(II)**) which show a significant improvement in cytotoxic activity toward HT-29 cells (IC_{50} = 0.004−1.51 μM). The most active compounds in this class were again those derived from 4-acetylpyrimidine (**4Cu(II)**, **5Cu(II)**, IC_{50} = 0.004 − <0.01 μM) and 2-acetyl pyrazine (**6Cu(II)** IC_{50} = <0.01 μM or **7**−**8Cu(II)**, IC_{50} = 0.004 − 0.028 μM). In general, complexation of the ligands **1**, **2**, **4**, and **6** with iron II results in a lowering of cytotoxic activity by a factor of ∼7. A slight improvement in the cytotoxic activity for HT-29 cells was observed for the 3-acetylpyridazine derived iron II complex (**1Fe(II)**, IC_{50} = 0.75 μM) by a factor of 2 as compared to **1Cu(II)** (IC_{50} = 1.51 μM). However **2Fe(II)** (IC_{50} = 0.77 μM) is less active than **2** (IC_{50} = 0.49 μM) and as active as **2Cu(II)** (IC_{50} = 0.87 μM).

Colony Forming Assay Determination of Compounds 5, 5Cu(II), 7, 7Cu(II), 8, and 8Cu(II). To obtain more detailed information about the antiproliferative activity, selected compounds were tested in a clonogenic assay using human tumor xenografts. An excellent correlation of drug response in clonogenic assays and in patients has been found.[21] The antiproliferative effects of the new compounds were tested in vitro in four human colorectal, one large cell lung, one small cell lung, two breast, two melanoma, two ovarial, two pancreas, and one renal tumor xenograft models (Table 2). Also in this assay, the compounds exhibited remarkable antiproliferative activity. The mean IC_{50} for **5**, **5Cu(II)**, **7**, **7Cu(II)**, **8**, and **8Cu(II)** were 6, 1, 0.18, 0.37, 1, and 0.37 nM, respectively (Table 2). It is interesting to note that the new compounds were particularly effective against human colon carcinoma (especially CXF 1103) and the large and small cell lung carcinoma xenografts LXFL 529 and LXFS 538. In contrast, the novel TSCs were less active in the following human tumor cells: mammary carcinoma MX1, melanoma MEXF 276L, renal carcinoma RXF 423, and the ovarial carcinomas. Between the most sensitive (LXFL 529) and the most resistant (OVRF 1353) tumor cells, the IC_{50} values differed by up to 840-fold.

In Vivo Antitumor Studies. In vivo antitumor studies using the large cell lung carcinoma LXFL529 were carried out with compound **5**. A combined dose finding/antitumor study at doses of 60, 30, and 10 mg/kg/day given i.p. on days 0, 4, and 8 in nude mice bearing this cell line was undertaken (Table 3). The highest dose (60 mg/kg/day) was clearly toxic with three deaths (75%) occurring after the first or second injection. Treatment with 30 mg/kg/day resulted in no toxic deaths; however, a body weight loss of 10% at the end of the study period (day 28) was observed. Doses of 10 mg/kg/d were well tolerated, and no signs of toxicity were observed. In a subsequent study, 40 mg/kg/d of compound **5** given on days 0, 4, and 8 resulted in the toxic deaths of 2 out of 4 mice. The results suggest that 30 mg/kg/d represents the maximal tolerable dose. With respect to the antitumor activity, application of 30 mg/kg/d resulted in a T/C (test/control) value of 58%,

Table 2. Inhibition of Cell Proliferation of Compounds **5**, **5Cu(II)**, **7**, **7Cu(II)**, **8**, and **8Cu(II)** in Colony-Forming Assays

human tumor xenograft		IC_{50}[a] (nM)					
		5	**5Cu(II)**	**7**	**7Cu(II)**	**8**	**8Cu(II)**
breast	MX1	149	14	3	5	18	4
	MCF7X	2	0.2	<0.1	0.21	0.35	< 0.1
colon	CXF158	16	2	0.19	0.28	1	0.l3
	CXF280	6	0.2	<0.1	<0.1	nd	< 0.1
	CXF1103	0.8	0.11	<0.1	<0.1	0.1	< 0.1
	HT29X	28	5	0.16	0.85	10	1
lung							
large cell	LXFL529	0.35	<0.1	<0.1	<0.1	0.16	< 0.1
small cell	LXFS538	0.24	0.19	0.15	0.22	0.28	0.18
melanoma	MEXF989	0.18	0.84	1	3	0.17	0.7
	MEXF276L	10	27	9	36	5	14
ovary	OVXF1023	9	15	5	16	4	10
	OVXF1353	294	19	25	25	100	24
pancreas	PAXF546	30	3	0.35	0.56	3	0.2
	PAXF736	27	15	<0.1	<0.1	< 10	< 10
renal	RXF423	28	26	9	16	6	4
mean IC_{50}, (nM)[b]		6	1	0.18	0.37	1	0.37

[a] Substance concentration required to reduce tumor cell colony formation to 50%. [b] Mean IC_{50} values were calculated according to the formula given in the Experimental Section.

Table 3. In Vivo Effects of Compound **5** given I.P. against the S.C.-Implanted Human Large Cell Lung Tumor Xenograft LXFL 529

compd	dose (mg/kg/inj)	schedule (days)	body wt change[a] (%)	presumed drug-related deaths	optimal T/C[b] (%)
control			+1		
5	10	0, 4, 8	−3	0	84
	30	0, 4, 8	−10	0	58
	60	0, 4	ne	3 of 4	ne

[a] Body weight changes are maximal losses or minimal gains expressed as a percentage of the initial body weight. [b] T/C = (median tumor volume of drug treated group/median tumor volume of the solvent treated control group) × 100. ne = not evaluable.

Table 4. Comparison of Inhibition of Cell Proliferation and [^{14}C]Cytidine Incorporation into DNA in Burkitt's Lymphoma Cells[a]

compd	IC_{50} (μM)		
	cell proliferation	[^{14}C]cytidine incorporation	ratio of IC_{50} values
hydroxyurea	140.000	37.15	0.26
5	0.022	2.10	95.45
5Cu(II)	0.018	0.21	11.66
7	0.006	0.32	53.33
7Cu(II)	0.006	0.18	30.00
8	0.011	3.32	301.81
8Cu(II)	0.006	2.97	495.00

[a] IC_{50} values for cell proliferation were determined in Burkitt's lymphoma cells. IC_{50} for [^{14}C]cytidine incorporation was determined in Burkitt's lymphoma cells as described in the Experimental Section. The data represent the means of two independent experiments in which duplicate determinations were taken within each experiment. The ratio of the IC_{50} values was calculated by IC_{50} cytidine incorporation/IC_{50} cell proliferation.

whereas at 10 mg/kg/d no influence on tumor growth was observed.

Incorporation of [^{14}C]Cytidine into DNA. To obtain information about the mechanism causing antiproliferative activity, we investigated the activity of RR following treatment with the new compounds. Incorporation of [^{14}C]cytidine into DNA was quantitated in intact Burkitt's lymphoma cells as a measurement of RR activity in situ. Table 4 shows that the compounds inhibited [^{14}C]cytidine incorporation, but in contrast to HU, the IC_{50} for inhibition of cell proliferation elicited by the new compounds is lower than that of [^{14}C]cytidine incorporation. The ratios of IC_{50} of [^{14}C]cytidine incorporation/IC_{50} cell proliferation are markedly different between HU and the new compounds (Table 4). This is an indication that the inhibition of DNA synthesis and the inactivation of RR does not seem to be the primary target of these acyl diazine-derived thiosemicarbazones.

Cell Cycle Analysis. Inhibitors of ribonucleotide reductase lead to a p53-independent arrest of cells in the S-phase (DNA synthesis) of the cell cycle.[22] Therefore, cell cycle analysis (arrest in S-phase) can indicate whether RR inhibition is involved in antiproliferative activity. For this reason, the newly synthesized compounds were tested for their ability to inhibit cell cycle progression. A concentration corresponding to the 2-fold IC_{50} of cell proliferation of the RR-inhibitor HU (IC_{50} = 140 μM) increases the percentage of Burkitt cells in S-phase after 48 h of treatment (Table 5). However, treatment with concentrations corresponding to 2-fold IC_{50} of cell proliferation of the new TSCs does not arrest cells in S-phase as does HU. As compared to the control, it can be concluded that these compounds are non-cell-cycle specific agents. This is a further indication that RR is not the primary target of the novel compounds.

Apoptosis. It has been shown that many antitumor agents are inducers of apoptosis. To obtain additional information about the mechanism of action, we quantitated the levels of apoptotic cells in Burkitt's lymphoma cells following treatment with the new compounds. Whereas compound **8Cu(II)** induces apoptosis to a similar extent as HU after 48 h, compounds **5**, **5Cu(II)**, and **8** are very potent inducers of apoptosis (Figure 2). Induction of apoptosis may at least in part be responsible for the antitumor activity elicited by some of these compounds at the low concentrations.

Metabolism of Compound 5 by Human Liver Microsomes. Although compound **5** exhibited high cytotoxic activity in the human tumor xenografts (mean IC_{50} is 6 nM) in vitro, its in vivo activity was rather poor. We proposed that the decreased in vivo activity may be due to the rapid biotransformation of compound **5** to inactive derivatives. Human liver microsomes were used as an in vitro model to investigate this phenomenon. Compound **5** was very rapidly metabolized to several not yet identified biotransformation products

Table 5. Cell Cycle Phase Distribution (%) of Hydroxyura (HU), and Compounds **5**, **5Cu(II)**, **7**, **7Cu(II)**, **8**, and **8Cu(II)** Treated Burkitt's Lymphoma Cells

	% of cells in different cell cycle phases (treated with 2 x IC_{50})					
	time (24 h)			time (48 h)		
compd	G1	S	G2-M	G1	S	G2-M
control	56.25 ± 2.23	19.98 ± 1.10	24.12 ± 1.15	61.30 ± 0.76	19.32 ± 0.88	17.67 ± 0.78
hydroxyurea	48.2 ± 2.03	38.22 ± 1.35	11.90 ± 0.86	21.27 ± 0.82	45.10 ± 0.67	19.52 ± 1.10
5	52.76 ± 2.34	23.41 ± 0.26	18.97 ± 2.41	55.47 ± 2.13	26.34 ± 3.53	20.36 ± 2.29
5Cu(II)	64.48 ± 1.26	21.70 ± 0.95	13.56 ± 0.76	59.19 ± 4.53	21.49 ± 4.11	11.40 ± 3.88
7	51.30 ± 2.06	23.79 ± 0.89	22.97 ± 1.56	49.20 ± 2.12	31.93 ± 0.88	19.81 ± 3.13
7Cu(II)	51.94 ± 1.65	22.82 ± 1.32	17.02 ± 6.13	49.33 ± 8.11	29.82 ± 1.76	9.81 ± 1.75
8	53.77 ± 4.02	22.57 ± 1.04	22.90 ± 4.37	54.14 ± 3.44	22.48 ± 1.30	19.44 ± 0.72
8Cu(II)	53.14 ± 3.17	25.91 ± 3.35	19.95 ± 0.83	52.73 ± 2.20	29.45 ± 2.91	17.66 ± 2.27

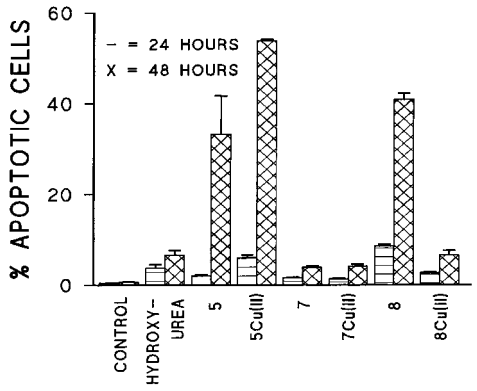


Figure 2. Induction of apoptosis by compounds **5**, **5Cu(II)**, **7**, **7Cu(II)**, **8**, and **8Cu(II)**. Burkitt's lymphoma cells were treated with concentrations corresponding to 2-fold IC_{50} of the compounds indicated. Apoptotic cells were quantitated as described in the Experimental Section. Data represent the means (± SD) of two independent experiments in which two samples were taken within each experiment.

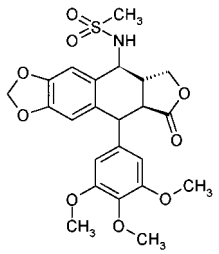


Figure 3. NSC 626315.

(data not shown) in that, 5 min after addition of compound **5** to the incubation medium, only 2% of unmetabolized compound could be quantified. As metabolite formation is strongly dependent on NADPH and as metabolic activity is inhibited by CO, cytochrome P450 isoenzyme(s) may catalyze the biotransformation of **5**.

Conclusions

The novel TSCs and their copper and iron complexes are very potent inhibitors of cell proliferation. Some of these inhibit cell growth in the nanomolar range (Table 1). In clonogenic assays with human tumor xenografts, the TSCs and their copper complexes exhibited potent cytotoxic activity in human colon carcinoma and large and small cell lung carcinoma cells. No inhibition was found in melanomas (except compound **8**), ovarial carcinomas, and renal carcinomas (Table 2). In a combined dose finding/antitumor study, application of compound **5** at a dose of 30 mg/kg/d resulted in a T/C value of 58%. This corresponds to 42% of tumor growth inhibition. A dose of 10 mg/kg/d exerted no influence on tumor growth, whereas doses of 40 and 60 mg/kg/d killed 50 and 75% of the treated mice (Table 3). In an in vitro metabolic study, compound **5** was found to be rapidly metabolized to yet unidentified products. This may be the reason the *in vivo* antitumor activity of this compound is very limited.

Our results also illustrate that the mechanism responsible for antitumor activity of the novel TSCs is not inhibition of RR. This conclusion is supported by the following results: (i) Inhibition of RR, as quantitated by incorporation of [^{14}C]cytidine into DNA occurs at high drug concentrations as compared to inhibition of cell proliferation. The ratio of IC_{50}-cytidine incorporation/ IC_{50}-cell proliferation of the novel compounds is not comparable to that of the RR inhibitor HU (Table 4). (ii) Cell cycle arrest in the S-phase induced by inhibitors of RR cannot be observed following treatment with the novel compounds (Table 5). Moreover, induction of apoptosis by some of these agents may be involved in the mechanism of action. We found that compounds **5**, **5Cu(II)**, and **8** are very potent inducers of apoptosis as compared to HU (Figure 2).

At the National Cancer Institute, compounds **1**, **4**, and **6** and the corresponding copper (II) and iron(II) derivatives were screened in vitro against a panel of 60 different human cell lines. Using compound **6** (NSC 339316) as a seed, analysis with the COMPARE algorithm[23] (a program that compares a complete set of cell sensitivities to those of standard agents or other agents present in the NCI database), showed a correlation coefficient of >0.6 to a number of thiosemicarbazones derived from acyl and benzoyl pyridines (Table 6). More surprisingly, a correlation coefficient of 0.797 was obtained for an analogue of etoposide (NSC 626315, Figure 3). Since a correlation coefficient of >0.6 can be considered as significant for this type of analysis, it is most probable that these types of TSCs may exert their antitumor activity partly through the inhibition of DNA topoisomerase II. This phenomenon has been partly substantiated in a recent publication[24] in which 2-acetyl pyridine TSCs were shown to be potent inhibitors of DNA topoisomerase II. However, it was observed that their mode of action was not to afford cleavable products as many topoisomerase II inhibitors and VP-16 do. Currently, investigations are underway to elucidate the mechanism by which the diazinyl-derived TSCs inhibit DNA topoisomerase II, and the results will be published in due course.

Table 6. Mean Panel GI_{50} Values and COMPARE Correlation Coefficients (PCC) Using Compound 6 (NSC 339316) as Seed, Tested in the U.S. National Cancer Institute (NCI) 60-Cell In Vitro Screen[a]

rank	NSC #	high conc	PCC	type of compd
1	339316	1.0E−04M	1.00	TSC analogue
2	319725	1.0E−04M	0.821	TSC analogue
3	668333	1.0E−04M	0.811	TSC analogue
4	668332	1.0E−04M	0.800	TSC analogue
5	319726	1.0E−04M	0.799	TSC analogue
6	626315	1.0E−04M	0.797	etoposide analogue
7	668301	1.0E−04M	0.795	TSC analogue
8	328785	1.0E−04M	0.791	TSC analogue
9	668336	1.0E−04M	0.781	TSC analogue
10	666159	1.0E−07M	0.776	discrete
14	635450	1.0E−06M	0.760	TSC (Cu complex)

[a] For definitions and methods of calculation of the correlation coefficient from the compare analyses, see ref 31.

In conclusion, although the TSCs studied are very potent inhibitors of cell proliferation, the in vivo antitumor results suggest that this series of compounds has low therapeutic index and can be considered as highly toxic. One significant factor is the rapid rate of metabolization and as such high efficacy in vivo activity may be achieved with derivatives less prone to metabolization.

Experimental Section

Chemistry. Infrared spectra (IR) were recorded from KBr pellets on a Mattson Galaxy Series FTIR 3000 spectrophotometer. NMR spectra were recorded from DMSO-d_6 solutions on a Varian Gemini 200 (^{1}H: 199.98 MHz and ^{13}C: 50.29 MHz) spectrometer. The center of the solvent signal was used as an internal standard which was related to TMS with δ 2.49 ppm (^{1}H) and δ 39.5 ppm (^{13}C). Assignments are based on chemical shift considerations as well as homonuclear NOE-difference experiments. Melting points were determined on a Reichert Thermovar hot stage microscope and are uncorrected. Elemental analyses were performed at the "Institut für Physikalische Chemie", University of Vienna, Austria and the data for C, H, N are within ± 0.4% of the calculated values. Reactions were monitored by TLC using Polygram SIL G/UV_{254} (Macherey-Nagel) plastic backed plates (0.25 mm layer thickness) and visualized using UV lamp. Column chromatography was performed using Kieselgel 60 (0.040−0.063 mm).

The following ketones utilized as starting materials were obtained according to the literature cited: methyl 3-pyridazinyl ketone (**9a**),[16] methyl 3-(6-methylpyridazinyl) ketone (**9b**),[17] ethyl 3-pyridazinyl ketone (**9c**),[16] methyl 4-pyrimidinyl ketone (**9d**),[17] methyl 4-(6-methylpyrimidinyl) ketone (**9e**),[18] methyl 2-(5methyl-pyrazinyl) ketone (**9h**).[19] Methyl 2-pyrazinyl ketone (**9f**) and methyl 2-(3-methylpyrazinyl) ketone (**9g**) were purchased from Maybridge Co.

General Procedure for the Synthesis of Methyl Hydrazinecarbodithioates 11e, 11g, and 11h. To a stirred solution of one equivalent of methyl hydrazinecarbodithioate (**10**) in 2-propanol was added one equivalent of the ketone **9e**, **9g**, or **9h**, respectively. The mixture was heated at 65−70 °C for 1 h and allowed to cool to room temperature. The product separating out after cooling at 4 °C overnight was filtered, washed with cold 2-propanol, and dried. The products obtained were used without further purification in the following reaction step. Analytically pure samples were obtained by additional recrystallization from ethanol.

Methyl 2-[1-(6-methyl-4-pyrimidinyl)ethylidene]hydrazinecarbodithioate (11e). Reaction of methyl hydrazinecarbodithioate (1.50 g, 12.27 mmol) with **9e** (1.68 g, 12.27 mmol) in 2-propanol (60 mL) gave 2.77 g (94%) of **11e** as yellow crystals. mp 152−154 °C; ^{1}H NMR δ 12.58 (s, 1H, NH), 9.08 (d, 1H, J = 1.3 Hz, pyrimidine H-2), 7.85 (s, 1H, pyrimidine H-5), 2.56 (s, 3H, SCH_3), 2.52 (s, 3H, 6-CH_3), 2.42 (s, 3H, acetyl-CH_3); ^{13}C NMR δ 12.4 (acetyl-$\underline{C}H_3$), 16.9 (6-$\underline{C}H_3$), 23.7 (S$\underline{C}H_3$), 115.4 (pyrimidine C-5), 149.4 (C=N), 157.8 (pyrimidine C-2), 160.9 (pyrimidine C-4), 166.8 (pyrimidine C-6), 201.4 (C=S). Anal. ($C_9H_{12}N_4S_2$) C, H, N.

Methyl 2-[1-(3-methyl-2-pyrazinyl)ethylidene]hydrazinecarbodithioate (11g). Reaction of methyl hydrazinecarbodithioate (1.50 g, 12.27 mmol) with **9g** (1.68 g, 12.27 mmol) in 2-propanol (60 mL) gave 2.89 g (98%) of **11g** as pale yellow crystals. mp 170−172 °C; ^{1}H NMR δ 12.48 (s, 1H, NH), 8.54 (m, 2H, pyrazine H-5/6), 2.82 (s, 3H, 3-CH_3), 2.54 (s, 3H, SCH_3), 2.48 (s, 3H, acetyl-CH_3); ^{13}C NMR δ 15.4 (acetyl-$\underline{C}H_3$), 17.1 (3-$\underline{C}H_3$), 24.7 (S$\underline{C}H_3$), 140.7* (pyrazine C-5), 142.8* (pyrazine C-6), 148.8 (pyrazine C-3), 153.1 (C=N), 152.3 (pyrazine C-2), 200.7 (C=S) (*interchangeable). Anal. ($C_9H_{12}N_4S_2$) C, H, N.

Methyl 2-[1-(5-methyl-2-pyrazinyl)ethylidene]hydrazinecarbodithioate (11h). Reaction of methyl hydrazinecarbodithioate (1.50 g, 12.27 mmol) with **9h** (1.68 g, 12.27 mmol) in 2-propanol (50 mL) gave 2.80 g (95%) of **11h** as off-white crystals. mp 167−168 °C; ^{1}H NMR δ 12.52 (s, 1H, NH), 9.10 (d, 1H, J = 1.4 Hz, pyrazine H-3), 8.54 (s, 1H, pyrazine H-6), 2.55 (s, 3H, 5-CH_3), 2.53 (s, 3H, SCH_3), 2.43 (s, 3H, acetyl-CH_3); ^{13}C NMR δ 12.5 (acetyl-$\underline{C}H_3$), 16.7 (3-$\underline{C}H_3$), 20.7 (S$\underline{C}H_3$), 140.9* (pyrazine C-6), 142.5* (pyrazine C-3), 146.9 (pyrazine C-5), 149.9 (C=N), 153.5 (pyrazine C-2), 200.7 (C=S) (*interchangeable). Anal. ($C_9H_{12}N_4S_2$), C, H, N.

General Procedure for the Synthesis of Thiosemicarbazone Derivatives 5, 7, and 8. To one equivalent of **11e**, **11g**, or **11h** (3.3 mmol) dissolved in hot methanol (30 mL) was added 3-azabicyclo[3.2.2]nonane (3.96 mmol). The mixture was heated under reflux until the evolution of methyl mercaptane had almost ceased. The resulting thiosemicarbazones crystallized out of the solution after cooling to room temperature. The crystals that had separated were collected by filtration and recrystallized from appropriate solvents (see Table 1).

N3-[(*E*-(1-(6-Methyl-4-pyrimidinyl)ethylidine]-3-azabicyclo[3.2.2]nonane-3-carbohydrazide (5). Compound **5** was obtained as yellow crystals in 98% yield. ^{1}H NMR δ 10.32 (br. s, 1H, NH), 9.05 (br. s, 1H, pyrimidine H-2), 7.78 (br. s, 1H, pyrimidine H-5), 4.10 (br. s, 4H, 2x N-CH_2-), 2.51 (s, 3H, 6-CH_3), 2.40 (s, 3H, acetyl-CH_3), 2.09 (br. s, 2H, 2× CH of bicyclo), 1.76−1.58 (m, 8H, C−CH_2 of bicyclo). Anal. ($C_{16}H_{23}N_5S$) C, H, N.

N3-[(*E*-(1-(3-Methyl-2-pyrazinyl)ethylidine]-3-azabicyclo[3.2.2]nonane-3-carbohydrazide (7). Compound **7** was obtained as light yellow crystals in 94% yield; ^{1}H NMR δ 9.68 (br. s, 1H, NH), 8.49−8.47 (m, 2H, pyrazine H-5/6), 4.05 (s, 2H, N−CH_2-), 4.03 (s, 2H, N−CH_2-), 2.79 (s, 3H, 3-CH_3), 2.37 (s, 3H, acetyl-CH_3), 2.06 (br. s, 2H, 2x CH of bicyclo), 1.73−1.55 (m, 8H, C−CH_2 of bicyclo). Anal. ($C_{16}H_{23}N_5S$) C, H, N.

N3-[(E-(1-(5-Methyl-2-pyrazinyl)ethylidine]-3-azabicyclo[3.2.2]nonane-3-carbohydrazide (8). Compound **8** was obtained as orange crystals in 90% yield; ^{1}H NMR δ 9.75 (br. s, 1H, NH), 8.98 (br. s, 1H, pyrazine H-3), 8.54 (s, 1H, pyrazine H-6), 4.06−4.05 (m, 4H, 2× N-CH_2,) 2.49 (s, 3H, 5-CH_3), 2.40 (br. s, 3H, acetyl-CH_3), 2.06 (br. s, 2H, 2× CH of bicyclo), 1.74−1.54 (m, 8H, C-CH_2 of bicyclo). Anal. ($C_{16}H_{23}N_5S$) C, H, N.

General Procedure for the Preparation of the Chloro-Cu(II) Complexes of the Thiosemicarbazone Derivatives 1−8. To a hot solution of 0.74 mmol of the acyldiazinyl thiosemicarbazones **1**−**8** in 30 mL of dry EtOH was added dropwise a solution of 0.13 g (0.74 mmol) of $CuCl_2 \cdot H_2O$ in 10 mL of dry EtOH. The mixture was heated at 40 °C for a further 4 h, the products separating out of the solution with time. The precipitates were collected by filtration after cooling the solutions to room temperature. The crystals were washed with cold EtOH, dried, and recrystallized. The elemental analyses were consistent for 1:1 complexes in the solid state. For yields, recrystalization solvents and physical data see Table 1.

General Procedure for the Preparation of the Sulfate-Fe(II) Complexes of the Thiosemicarbazone Derivatives 1−8. The thiosemicarbazone ligands **1**−**8** (1 mmol) were dissolved in 50 mL of dry EtOH by warming. After the solution was allowed to cool to room temperature, 1 mmol of $FeSO_4 \cdot$

$7H_2O$ dissolved in 10 mL of dry EtOH was added dropwise. The mixture was stirred at room-temperature overnight with the exclusion of moisture. The products precipitating out were filtered, washed with cold EtOH, and dried. In cases where no precipitate was formed, the solution was evaporated to dryness. The products obtained were recrystallized from appropriate solvents. For yields, recrystallization solvents and physical data see Table 1. The elemental analyses were consistent for 1:1 complexes in the solid state.

Biological Methods. (a) Cytotoxicity Assays. Burkitt's lymphoma (CA 46, ATCC CRL 1648) and CCRF-CEM (acute lymphoblastic leukemia, ATCC CCL 119) cells were grown in RPMI 1640 medium. HT-29 (colon adenocarcinoma cells, ATCC HTB 38) were grown in McCoy's 5A medium. The media were supplemented with 10% fetal calf serum (except Burkitt's lymphoma with 15%), 2 mM glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin. Approximately 10 000 cells were seeded per well into 96-well plates. The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to the final concentration with growth medium. The concentration of DMSO was up to 0.5%, and this was not toxic. After an initial incubation period of 4 h, various concentrations of the test compounds were added to the cells and exposed in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 72 h. Dose−response curves for CCRF-CEM and Burkitt's lymphoma cells were detected by the MTT-assay[25] from Boehringer Mannheim, Mannheim, Germany. Inhibition of cell proliferation of HT-29 cells was detected by the SRB-assay.[26] Subsequently, the samples were processed and absorption detected by a microplate reader (model 3550, Bio-Rad, Hercules, CA). The IC_{50} values were calculated by the CalcuSyn software from Biosoft, Cambridge, UK.

(b) Clonogenic Assays. A modification of the double-layer soft agar assay was used.[27] Solid human tumor xenografts growing s.c. in the athymic nude mice were removed, mechanistically disaggregated, and subsequently incubated with a disaggregating solution consisting of collagenase (1.2−1.8 U/mL), DNase (375 U/mL), and hyaluronidase (29 U/mL) in RPMI 1640 medium at 37 °C for 30 min. The cell mixture was washed twice with phosphate buffered saline, and passed through sieves of 200 and 500 μM mesh size, and the percentage of viable cells was determined in a Neubauer counting chamber using trypan blue exclusion. A total of 8×10^3 to 1.6×10^4 cells in 0.2 mL of Iscove's modified Dulbecco's medium with 20% fetal calf serum and 0.4% agar were plated onto a base layer consisting of 0.2 mL of the same culture medium and 0.7% agar in 24-well plates. The compounds were applied 1 day after cell seeding (continuous exposure). Every plate contained six controls receiving the vehicle only, and drug-treated groups were plated in triplicate in six concentrations ranging from 0.1 mM to 10 μM. Cultures were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 6−18 days (depending on the doubling times of the tumor stem cells) and monitored closely for colony growth using an inverted microscope. At the time of maximum colony formation (colony diameter > 50 mm), counts were performed with an automatic image analysis system OMNICON FAS I (Bausch & Lomb V). Twenty-four hours before evaluation, vital colonies were stained using a tetrazolium chloride dye.

Compound effects were expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies in the treated plates with the mean colony count of the untreated controls (colony count T/C × 100). Mean IC_{50} values were calculated according to the formula:

$$\text{mean } IC_{50,70} = 10^{\frac{\sum_{x=1}^{n} \log(IC_{50,70})x}{n}}$$

where x = specific tumor xenograft and m = total number of xenografts studied and IC_{50} values = substance concentration required to reduce tumor cell colony formation to 50%.

In Vivo Test Procedures. Tumor fragments harvested from the subcutaneously growing human large cell lung carcinoma LXFL 529 in nude mice hosts were implanted subcutaneously into both flanks of six- to eight-week-old female athymic nude mice of the Balb/C strain homozygous for the nude gene (nu/nu). When tumors were approximately 5−7 mm in diameter, mice were randomly assigned to treatment groups and untreated controls. The control group consisted of 4 mice bearing 6 evaluable tumors, and the drug treated groups consisted of 3−4 mice bearing 4−5 evaluable tumors. Compound **5** was applied as a fine suspension in sterile water containing 2.5% DMSO and 2.5% polysorbate 80 on days 0, 4, and 8 at doses of 60, 30, and 10 mg/kg/day. Mice were weighed and tumors were measured using calipers twice weekly. Tumor volume was calculated according to the formula: Tumor volume (mm^3) = Width $(mm)^2$ × length (mm)/2. Data evaluation was performed using software developed in our laboratory. Relative tumor volume (RTV) values were calculated for each single tumor by dividing the tumor volume day X (TV_x) by the tumor volume on day 0 (TV_0) at the time of randomization [$RTV = (TV_x \text{ X } 100/TV_0)$]. Median RTV values were used for further evaluation. Additionally, T/C values were calculated by comparing the relative tumor size of treated groups and controls.

Incorporation of [^{14}C]Cytidine into DNA. Incorporation of [^{14}C]cytidine into DNA was employed as an indicator for inhibition of ribonucleotide reductase. The assay was performed by a modification of the procedure previously described[28] in intact Burkitt's lymphoma cells. 3 mL ($(1-2) \times 10^6$/mL) of exponentially growing cells were incubated (37 °C) in medium with various drug concentrations of each compound. After 90 min, 0.7 μCi [^{14}C]cytidine (2−10 Ci/mmol, ICN Biomedicals, Meckenheim, Germany) were added to each sample. Subsequently, the cells were washed twice with ice-cold phosphate buffered saline, resuspended in 1 mL of ice-cold trichloroacetic acid, and transferred to Eppendorf tubes. Following an incubation period on ice for 20 min, the disrupted cells were centrifuged at 3000*g* for 5 min (+4 °C). The pellet was resuspended in 800 μL of 80 mM Tris-HCl (pH 8) to which 20 μL DNase-free RNase (10 mg/mL) was added. Following a 2-h incubation at 37 °C, the solution was cooled on ice, and 200 μL of 50% trichloroacetic acid was added. The solution was incubated at +4 °C overnight. The trichloroacetic acid-precipitable material was obtained by centrifuging at 4000*g* (+4 °C) for 5 min. The pellet was washed four times in 1 mL of ice-cold 5% trichloroacetic acid, dissolved in 0.4 mL of 1.25 M NaOH solution overnight, and counted in 2 mL of scintillation fluid (Ultima Gold, Packard, Meriden, CT) to evaluate the DNA-incorporated [^{14}C]cytidine.

Cell Cycle Analysis and Apoptosis. Cell cycle analysis was performed as described previously.[29] The cells were treated with concentrations corresponding to the 2-fold IC_{50} of the compounds for 24 and 48 h. Subsequently, 250 μg/mL propidium iodide (Sigma, Vienna, Austria) dissolved in 5% Triton-100 and 1 mg/mL RNase A (Sigma) were added. Following incubation of the sample at room temperature for 1 h, the cell cycle was analyzed by a FACscan (Becton Dickinson, Mountain View, CA). In the same experiment, the percentage of apoptotic cells was quantitated by analysis of the hypodiploid DNA peak as described by Nicoletti et al.[30]

Metabolic Studies of Compound 5. Pooled human liver microsomes (Gentest, Woburn, MA, USA; 0.4 mg of protein/mL), NADPH (1 mM), isocitric acid (5 mM), and isocitric dehydrogenase (Sigma, Vienna, Austria) were preincubated at 37 °C in 0.1 M phosphate buffer, pH 7.4 (final volume 250 μL). The reaction was started by the addition of compound **5** (final concentration 100 μM) in DMSO (to yield an incubation DMSO concentration of not >0.75% v/v), and the metabolism was assessed from 0 to 60 min. The reaction was stopped by the addition of 500 μL of methanol, samples were centrifuged (10000*g*, 5 min), and 40 μL of the clear supernatant was injected onto the HPLC column. HPLC was performed using a Shimadzu liquid chromatograph consisting of a LC-6A pump, a SIL-6B autoinjector and a SPD-6AV UV−Vis detector that was set at a wavelength of 238 nm. A Nina data system (Nuclear Interface, Muenster, Germany) determined retention

times and peak areas. Chromatographic separations were performed on a Hypersil BDS-C18 (5 μM, 250 × 4.6 mm; 3 mm i.d., Astmoor, England) preceded by a Hypersil BDS-C18 precolumn (5 μM, 10 × 4.6 mm i.d.) at a flow rate of 1.0 mL/min. The mobile phase consisted of heptanesulfonic acid (5 mM) in potassium phosphate (50 mM, pH 4.0, with phosphoric acid) and methanol (6:4, v/v).

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