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Studies on the Side-Chain Hydroxylation of Ifosfamide and Its Bromo Analogue

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Abstract—Deutero-substituted (α,α,α′,α′-tetradeuterated) derivatives of ifosfamide (IF-d_4) and its bromo analogue were synthesised. In vitro metabolic studies showed that microsomal hydroxylation of IF-d_4 is slower than for unlabelled compound, suggesting that kinetic isotope effect operates during those transformations. At the same time deutero-substituted derivatives are more active against L1210 leukaemia in mice than unlabelled compounds, suggesting a negative role of side-chain hydroxylation metabolic pathways in the anticancer activity of ifosfamide and its analogues.

In spite of extensive studies on antitumour agents, including new approaches like gene therapy, alkylating agents[1] are still the most widely used cytostatics to combat various types of cancer. In the last decade ifosfamide {[*N*,3-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide], IF} proved to be an effective drug in clinical oncology.[2] However, therapeutic application of high-dose IF is limited by several side effects; among them neurotoxicity[3] and nephrotoxicity[4] are of the greatest concern. Based on the analysis of metabolic pathways of IF in humans[5] (Scheme 1) it was postulated that these side effects are connected with P450 cytochrome catalysed bio-oxidation of IF and the release of chloroacetaldehyde as the result of hydroxylations at C-1 atoms of 2-chloroethyl groups.[3]

At the same time, hydroxylation of C-4 atom of 1,3,2-oxazaphosphorine ring is essential for IF activity since this metabolic pathway leads to the final cytotoxic metabolite isophosphoroamide mustard (iPAM). Compounds that have an ability to modulate cytochrome P450 oxidation activity, for example methylene blue, were clinically used to reverse neurotoxicity of IF.[6] On the other hand, Wagner postulated recently[7] that chloroacetaldehyde may play a positive role in the anticancer activity of this drug. In the search for more effective ifosfamide congeners it is important to assess the influence of side-chain hydroxylations on anticancer activity of this class of compounds. The level of chloroacetaldehyde produced during IF biotransformation should be affected by introduction of deuterium atoms at the sides of hydroxylation since kinetic isotope effect ought to operate[8] during these reactions.

In our previous studies we found that bromo analogues of IF are potent anticancer agents.[9] Similarly as for stereoisomers of cyclophosphamide and ifosfamide,[10] also among examined bromo analogues of IF (−) enantiomers are more potent than (+) enantiomers and racemates against several experimental tumours in mice.[11] Based on those results (*S*)-(−)-3-(2-bromoethyl)-*N*-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide[12] [(*S*)-(−)-BF] became the subject of phase I clinical trials in Poland. Obtained preliminary data[13] showed that (*S*)-(−)-BF possesses similar profiles of side effects as IF. Therefore studies on the influence of deuterium atom introduction in both (*S*)-(−)-BF and racemic BF on their anticancer activity were also undertaken.

At first {[*N*,3-bis(2-chloro-1,1-dideuteroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide] (IF-d_4)} was synthesised (Scheme 2).

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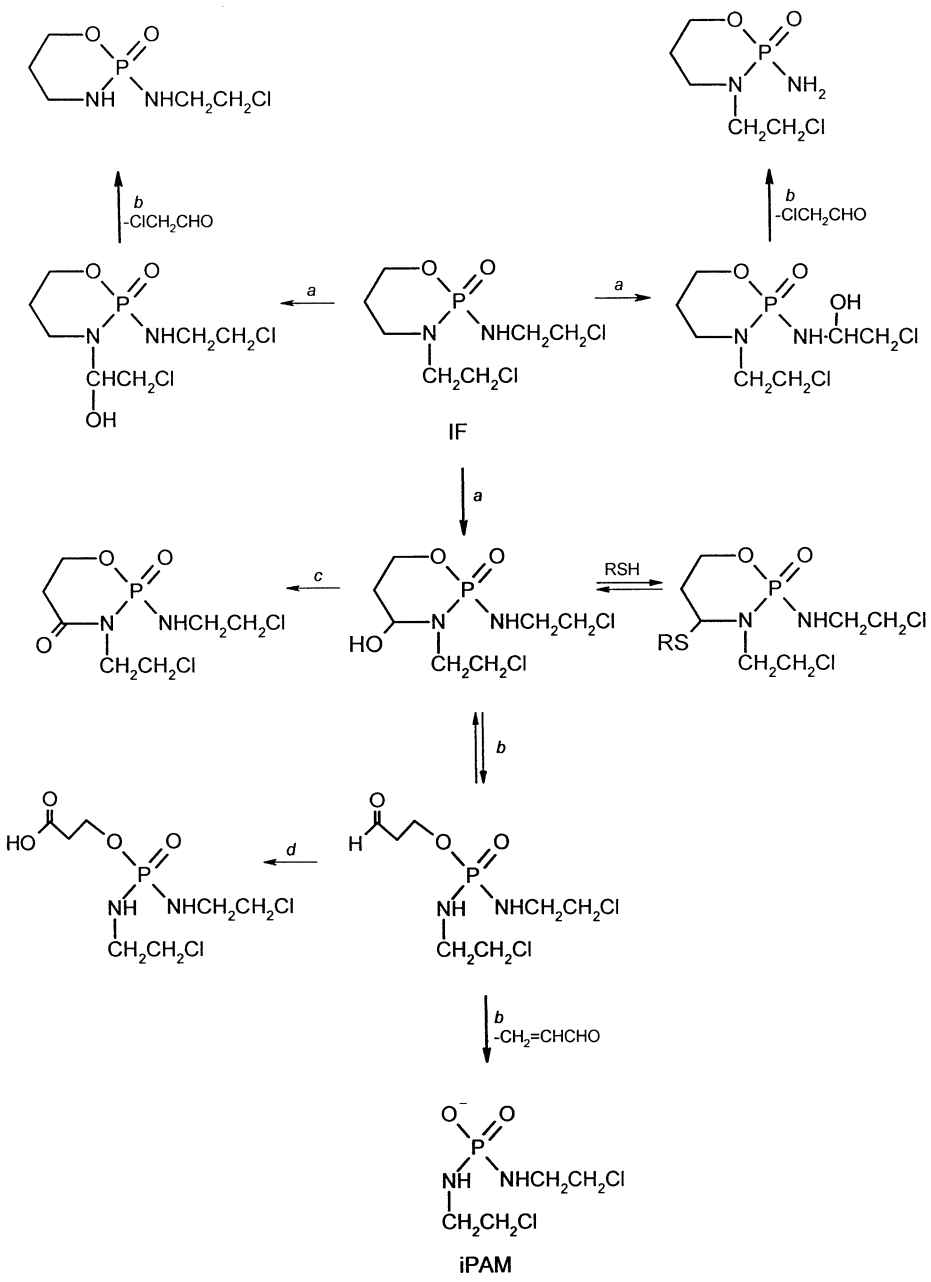
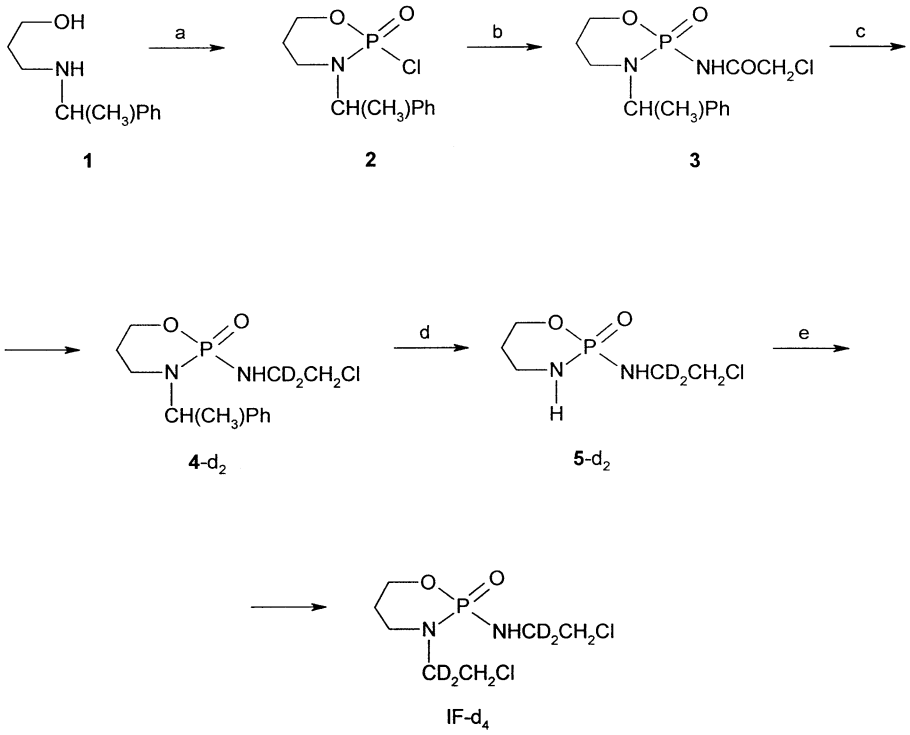


Scheme 1. (a) Cytochrome P450; (b) spontaneous; (c) mechanism unknown; (d) aldehyde dehydrogenase.

Introduction of deuterium atoms was performed using chemistry of incorporation of 2-chloroethyl groups into tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine moiety by halogenoacetylation and subsequent reduction of carbonyl group.[14] An early attempt to introduce two 2-chloroacetyl groups in one step was unsuccessful, so processes of acylation of *exo*cyclic and *endo*cyclic nitrogen atoms were separated. Starting from racemic α-methylbenzylamine and 3-chloropropanol, amino-alcohol **1** was obtained,[14] which was condensed with $POCl_3$ in the presence of triethylamine giving single diastereoisomer of chlorophosphate **2**.[15] Compound **2** was subject to ammonolysis and subsequent chloro-acetylation. In obtained **3**,[16] amidic hydrogen was exchanged into deuterium atom by the action of methanol-d_1 (monitored by ^{1}H NMR). It was found that without this step subsequent reduction of carbonyl group into methylene moiety by sodium borodeuteride (98% D) in the presence of $BF_3{\cdot}Et_2O$ leads to substantial loss of deuterium atoms in the product. In obtained **4**-d_2, α-methylbenzyl N-3 protecting group was removed by hydrogenolysis in the presence of palladium on activated carbon providing intermediate **5**-d_2.

Repeating chloroacetylation/reduction reactions gave desired IF-d_4 (mp 49–50 °C) in 6% overall yield. The deuterium content established by mass spectrometry was 92% of compound d_4 and 7% of d_3.

Using bromoacetyl bromide in the presence of Et_3NHBr for acylation of **5**-d_2 and employing the same method of synthesis as presented above, 3-(2-bromo-1,1-dideutero-ethyl)-*N*-(2-chloro-1,1-dideuteroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide (BF-d_4) (mp



Scheme 2. (a) $POCl_3$, Et_3N; (b) (1) NH_3; (2) $ClCH_2COCl$, Et_3NHCl; (c) (1) MeOD; (2) $NaBD_4$, $BF_3{\cdot}Et_2O$; (3) MeOH; (d) H_2, Pd/C; (e) (1) $ClCH_2COCl$, EtNHCl; (2) $NaBD_4$, $BF_3{\cdot}Et_2O$.

63–64 °C) was obtained in 5% total yield. Starting from (−)-α-methylbenzylamine and using the method employed for the synthesis of unlabelled (*S*)-(−)-bromofosfamide,[12] the (*S*)-(−)-BF-d_4 [mp 84–85 °C; $[\alpha]_D = -41.0$ (*c* 2.0, MeOH)] was prepared. Enantiomeric purity of obtained (*S*)-(−)-BF-d_4 was 100% as proved by recording its ^{31}P NMR spectrum in the presence of $Eu(tfc)_3$[14] [$CDCl_3$ solution, ratio (*S*)-(−)-BF-d_4/$Eu(tfc)_3$ 1:0.7].

Synthesis of IF-d_4 enabled studies on the presence of the kinetic isotope effect operating during microsomal hydroxylation of C-1 atoms of 2-chloroethyl groups in ifosfamide. A mixture of IF and IF-d_4 (1:1 ratio) was incubated in the presence of rat liver microsomes, β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase in conditions previously established for in vitro activation of oxazaphosphorine drugs.[17] The incubation[18] was stopped after 5, 10, 20 and 50 min and cyclophosphamide (CP) as internal standard was added into reaction mixture. IF, IF-d_4, and CP were extracted with ethyl acetate and then derivatised by the addition of trifluoroacetic anhydride. Percentage of activation of IF and IF-d_4 and their ratio were established using GC–MS analysis[19,20] (Table 1).

Maximum extent of in vitro metabolism was 65% and was achieved after 20 min. The ratio of IF to IF-d_4 was lowered together with the progress of reaction, suggesting the existence of a kinetic isotope effect in processes of side-chain hydroxylation of ifosfamide. Another, however less likely, possibility is that IF-d_4 is a poorer substrate than unlabelled compound for cytochrome P450 due to steric effect caused by larger deuterium atoms.

Synthesised racemic deutero-substituted IF and both racemate and (−) enantiomer of its bromo analogue were tested for their anticancer activity in vivo.[21] IF, BF, and (*S*)-(−)-BF were used as the reference compounds. All tested agents were administered ip in a single-dose treatment schedule to CD2F1 mice bearing advanced (day 3) ascitic L1210 leukaemia. Increase in life span (ILS) of treated mice over control was calculated according to the formula: T/C ×100 − 100, where T and C are average survival times (AST) of treated and control mice, respectively. It was found that in all cases deutero analogues were more active than unlabelled standards (Fig. 1).

Stereodifferentiation of anticancer activity of deutero-substituted bromo analogues of ifosfamide that favours (−) enantiomer was also observed. Sub-acute toxicity[22] of the most potent agent (*S*)-(−)-BF-d_4 was compared

Table 1. Relative rates of microsomal[a] metabolism of IF and IF-d_4

Time of incubation (min)	Extent of metabolism (%)	IF/IF-d_4 ratio
0[b]	0	1.000
5	29	0.974
10	51	0.948
20	65	0.910
50	65	0.908

[a]Microsomes were isolated from rat liver.
[b]Reference sample with inactive (thermally denaturated) microsomes.

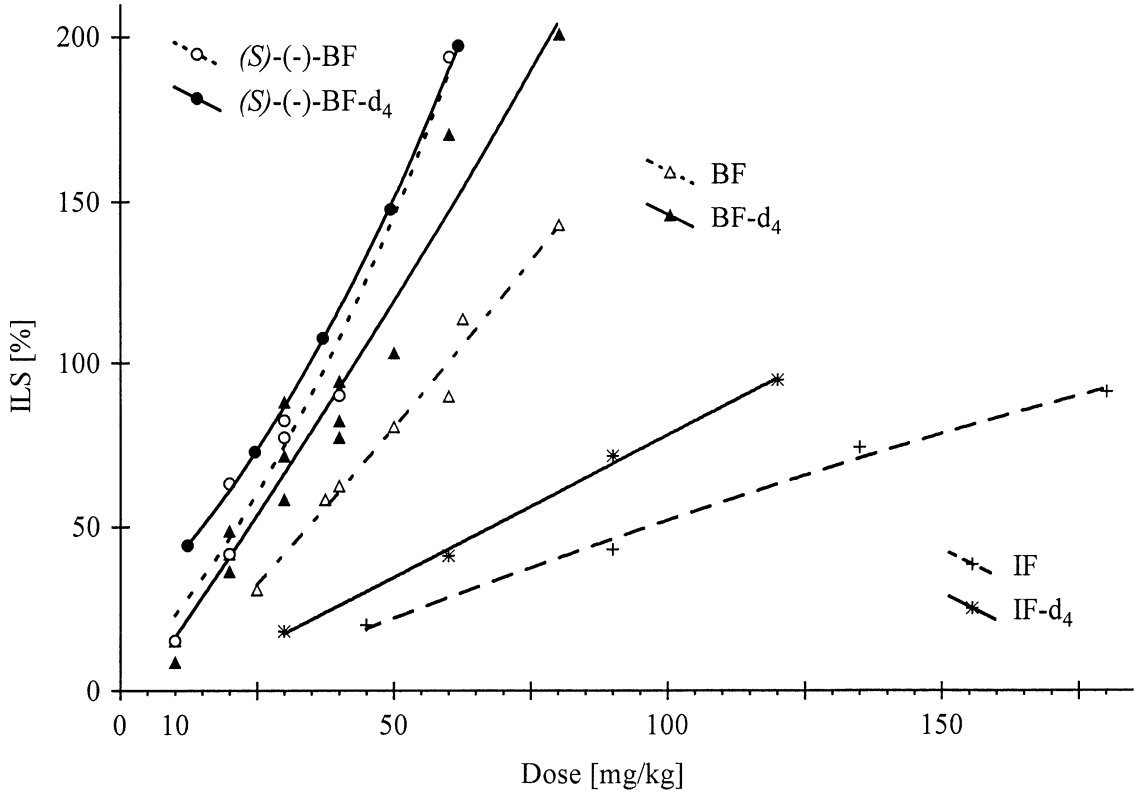


Figure 1. Antitumour activity of deutero-substituted ifosfamide and its bromo analogues against L1210 leukaemia in mice.

with toxicity of unlabelled compound and they were found to be nearly the same. As a result of the high potency of (*S*)-(−)-BF-d_4 this agent possesses therapeutic index much higher than those calculated for (*S*)-(−)-BF, BF, or IF (Table 2).

Studies on neurotoxic effects of obtained deutero analogues of IF, BF and (*S*)-(−)-BF in mice are now in progress.

It can be concluded that revealing that deutero-substituted compounds possess higher anticancer activity than unlabelled standards suggests a negative role of side-chain hydroxylation metabolic pathways for biological activity of this group of compounds. Based on this assumption new methyl analogues of ifosfamide with the decreased possibility of side-chain hydroxylation were synthesised.[23] However, their anticancer activities against L1210 leukaemia in mice were lower than for ifosfamide, probably due to a lower ability to cross-link DNA by their final, active metabolites. Another possibility to increase therapeutic efficacy of IF or its bromo analogues is to selectively block these subtypes of cytochrome P450 that are responsible for side-chain hydroxylation. Very recently Waxman showed[24] that CYP3A4 mostly catalyses C-4 atom hydroxylation while CYP2B6 hydroxylates C-1 atoms of 2-chloroethyl groups of IF.

Table 2. Antitumour effectiveness of deutero-substituted derivatives of ifosfamide and bromofosfamide against L1210 leukaemia in mice

Compd	ED_{50} (mg/kg)	LD_{50}[a] (mg/kg)	MTD (mg/kg)	TI[a]
IF	96.4	687 (632–746)[b]	518	7.1
IF-d_4	67.8	N.D.[c]	N.D.	
BF	34.3	320 (302–339)	236	9.3
BF-d_4	23.6	N.D.	N.D.	
(*S*)-(−)-BF	21.3	289 (276–303)	230	13.6
(*S*)-(−)-BF-d_4	15.0	265 (242–290)	196	17.7

[a]$TI = LD_{50}/ED_{50}$.
[b]Confidence limits.
[c]N.D., not determined.

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References and Notes

1. Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723.
2. (a) Johnson, D. H. *Semin. Oncol.* **1996**, *23* (Suppl. 6), 7. (b) Prat, C. B. *Semin. Oncol.* **1996**, *23* (Suppl. 6), 84. (c) O'Byrne, K.; Steward, W. P. *Oncology* **1999**, *56*, 13.
3. Lewis, L. D.; Meanwell, C. A. *Lancet* **1990**, *335*, 175.
4. Skinner, R.; Sharkey, I. M.; Pearson, A. O.; Graft, A. W. *J. Clin. Oncol.* **1993**, *11*, 173.
5. (a) Misiura, K.; Okruszek, A.; Pankiewicz, K.; Stec, W. J.; Czownicki, Z.; Utracka, B. *J. Med. Chem.* **1983**, *26*, 674. (b) Boddy, A. V.; Yule, S. M. *Clin. Pharmacokinet.* **2000**, *38*, 291. (c) Kerbusch, T.; de Kraker, J.; Keizer, J.; van Putten, J. W. G.; Groen, H. J. M.; Jansen, R. L. H.; Schellens, X.; Beijnen, J. H. *Clin. Pharmacokinet.* **2001**, *40*, 41.
6. Kupfer, A.; Aeschlimann, C.; Wermuth, C. B.; Cerny, T. *Lancet* **1994**, *343*, 763.

7. (a) Bruggmann, S. K.; Kisro, J.; Wagner, T. *Cancer Res.* **1997**, *57*, 2676. (b) Borner, K.; Kisro, J.; Bruggemann, S. K.; Hagenah, W.; Peters, S. O.; Wagner, T. *Drug Metab. Dispos.* **2000**, *28*, 573.
8. Higgins, L.; Bennett, G. A.; Shimoji, M.; Jones, J. P. *Biochemistry* **1998**, *37*, 7039.
9. Misiura, K.; Kinas, R. W.; Stec, W. J.; Kuśnierczyk, H.; Radzikowski, C.; Sonoda, A. *J. Med. Chem.* **1988**, *31*, 226.
10. Kuśnierczyk, H.; Radzikowski, C.; Paprocka, M.; Budzyński, W.; Rak, J.; Kinas, R. W.; Misiura, K.; Stec, W. J. *J. Immunopharmacol.* **1986**, *8*, 455.
11. Glazman-Kuśnierczyk, H.; Matuszyk, J.; Radzikowski, C. *Immunopharmacol. Immunotoxicol.* **1992**, *14*, 883.
12. Misiura, K.; Kinas, R. W.; Kuśnierczyk, H.; Radzikowski, C.; Stec, W. J. *Anti-Cancer Drugs* **2001**, *12*, 453.
13. Kobylińska, K.; Koralewski, P.; Sobik, B.; Gasiorek, X.; Kobylińska, M. *Arzneim. Forsch. Drug Res.* **2001**, *51*, 600.
14. Pankiewicz, K.; Kinas, R.; Stec, W. J.; Foster, A. B.; Jarman, M.; Van Maanen, J. M. S. *J. Am. Chem. Soc.* **1979**, *101*, 7712.
15. Sato, T.; Ueda, H.; Nakagawa, K.; Bodor, N. *J. Org. Chem.* **1983**, *48*, 98.
16. All new compounds were fully characterised with ^{1}H and ^{31}P NMR and with MS.
17. (a) Connors, T. A.; Cox, P. J.; Farmer, P. B.; Foster, A. B.; Jarman, M. *Biochem. Pharmacol.* **1974**, *23*, 115. (b) Foster, A. B.; Jarman, M.; Kinas, R. W.; van Maanen, J. M.; Taylor, G. N.; Gaston, J. L.; Parkin, A.; Richardson, A. C. *J. Med. Chem.* **1981**, *24*, 1399.
18. Solutions of IF-d_4 or IF (0.4 mM, 10 mL) in 0.1 M Tris–HCl, pH 7.4, containing: 5 mM $MgCl_2$, 6 mM glucose-6-phosphate, 0.3 mM NADP, glucose-6-phosphate dehydrogenase (0.8 U/mL) and active or inactive microsomes equivalent to 1 g of rat liver were incubated in oxygen atmosphere at 37 °C for 5, 10, 20 and 50 min. After each time a sample was removed (2 mL) and chilled in ice. After addition of the 0.4 mM solution of cyclophosphamide in 0.1 M Tris–HCl, pH 7.4 (0.2 mL), each sample was extracted with ethyl acetate (3 × 3 mL). Organic solutions were combined, dried with Na_2SO_4, concentrated and redissolved in ethyl acetate (0.2 mL), mixed with trifluoroacetyl anhydride (0.2 mL), and heated at 80ºC for 20 min. After that time solutions were cooled down, concentrated and redissolved in ethyl acetate (100 μL). Obtained samples were analysed on GC–MS.
19. Fenselau, C. *Anal. Chem.* **1977**, *49*, 563A.
20. Sample (5 μL) was injected into OV 17 column (3%, Chromosorb Q, 2.7 m) working at 240 °C.
21. Antitumour effect of examined agents was evaluated in vivo after single-dose treatment (day 3 only) of mice bearing L1210 leukaemia. CDF1 mice were inoculated ip with 10^5 ascitic tumour cells suspended in 0.2 mL of PBS. The experiments were conducted and activity of tested compounds was evaluated according to the NIH/NCI in vivo standard screening protocols. Effective doses providing 50% increase of lifespan of treated animals over control (ED_{50}) were estimated graphically from the least square-fitted dose–effect curves.
22. Sub-acute toxicity (45-day observation period) of compounds IF, BF, (*S*)-(−)-BF and (*S*)-(−) BF-d_4 was evaluated in healthy CD2F1 females, 12–16 weeks old and weighing 25–27 g. Mice were injected once by ip route or were given one oral gavage (po route) of tested compounds. Mortality distribution was cumulated from three to six separate experiments in which usually four to seven dose levels were used, with at least five mice in a group. The log/dose–expected effect curves were constructed according to Litchfield–Wilcoxon method, based on the mortality distribution during observation period. Doses lethal for 50% of animals (LD_{50}) as well as LD_5 maximally tolerated dose (MTD) were estimated graphically from the constructs and 95% confidence limits were calculated for LD_{50}. All computations were performed using CSS Statistica software (StatSoft).
23. Misiura, K.; Kardacka, K.; Kuśnierczyk, H. *Arch. Pharm. Pharm. Med. Chem.* **2001**, *334*, 291.
24. Huang, Z.; Roy, P.; Waxman, D. J. *Biochem. Pharmacol.* **2000**, *59*, 961.