# Synthesis of 7-Benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine and Novel Derivatives Free of Positional Isomers. Potent Inhibitors of cAMP-Specific Phosphodiesterase and of Malignant Tumor Cell Growth

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7-Benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine (**7a**) is a potent inhibitor of the cAMP-specific phosphodiesterase isoenzyme family PDE4 and induces growth inhibition in a panel of tumor cell lines. In this study, we describe a synthesis that yields **7a** and novel derivatives free of positional isomers. The synthesis of alkylamino substituted pteridines is based on the successive nucleophilic aromatic substitution of the chlorine atoms of 2,4,6,7-tetrachloropteridine. For the reaction with secondary amines, the positional order of reactivity was found to be C4 > C7 > C2 > C6. Final structural proof is given by X-ray crystallography. To unravel structural elements of **7a** crucial for the interaction with the target enzyme, the compound was modified systematically. The impact of the modifications on activity was tested by evaluating the ability of the compounds to inhibit cAMP hydrolysis by cAMP-specific phosphodiesterase (PDE4) purified from the solid human large cell lung tumor xenograft LXFL529. Growth inhibitory properties were determined by in vitro treatment of the respective cell line LXFL529L using the sulforhodamine B assay (SRB). The results show that for high activity, the heterocyclic substituent in position 2 of the pteridine ring system requires the presence of a basic nitrogen in 4′-position, as represented by piperazine.

## Introduction

The second messenger cyclic adenosine monophosphate (cAMP) is known to play a pivotal role in the regulation of cell growth and differentiation. Hydrolysis of cAMP, resulting in the formation of 5′-AMP, is catalyzed by phosphodiesterases (PDEs), a superfamily of isoenzymes. According to substrate specificity and sensitivity to endogenous modulators, at present there are at least nine different PDE isoenzyme families known.[1] In the last years, enormous progress has been achieved to elucidate the complex expression patterns of PDEs in different cell types and tissues. Nevertheless, only limited information is available concerning PDE patterns of malignant cells. In the murine melanoma B16, the murine spindle cell carcinoma CarB, and the human mammary carcinoma MCF-7, the cAMP-specific rolipram-sensitive isoenzyme family PDE4 has been shown by us to represent the highest cAMP hydrolyzing activity.[2,3] Treatment of these cell lines with the specific PDE4 inhibitor 7-benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine (**7a**) induces dose-dependent growth inhibition.[2,3]

Human PDE4 is encoded by four different genes, the catalytic domain being highly conserved. Since crystal structures of PDE4 and PDE4 inhibitor complexes are not yet available, knowledge about structural requirements for interaction of potential inhibitors with the enzyme is limited. To get more insight into potential sites of interaction with the target enzyme, structural elements of the highly effective PDE4 inhibitor **7a** were modified systematically. The impact of the modifications was evaluated by testing the ability of the compounds to inhibit cAMP hydrolysis of PDE4 purified from solid tumor tissue of the human large cell lung tumor xenograft LXFL529, a tumor which we have found to express very high PDE activity in both solid tumor tissue grown in nude mice as well as in cell culture. About 80% of overall PDE activity of LXFL529 was represented by PDE4; very minor additional activities (≤10%, respectively) could be ascribed to PDE1 and PDE3 (data not shown). Treatment-induced growth inhibitory properties were determined with the sulforhodamine B assay (SRB).

## Chemistry

The synthesis of **7a** and its 4,7-bis(dimethylamino) analogue **7f** has been described already in patents[4,5] without addressing, however, the formation of positional isomers generated when the described procedure is followed. The present paper describes an approach to obtain alkylamino substituted chloropteridines free of positional isomers. Since with some of these compounds unequivocal structural proof was not achievable by $^{1}H$ and $^{13}C$ NMR spectroscopy, X-ray crystallography was used for structure characterization of two pivotal representatives.

The synthesis of alkylamino substituted pteridines is based on the successive nucleophilic aromatic substitution of the chlorine atoms of 2,4,6,7-tetrachloropteridine at different temperatures and in various solvents. Following the synthesis instructions of the patent literature,[4,5] the sequence of substitutions is believed to occur according to Scheme 1.

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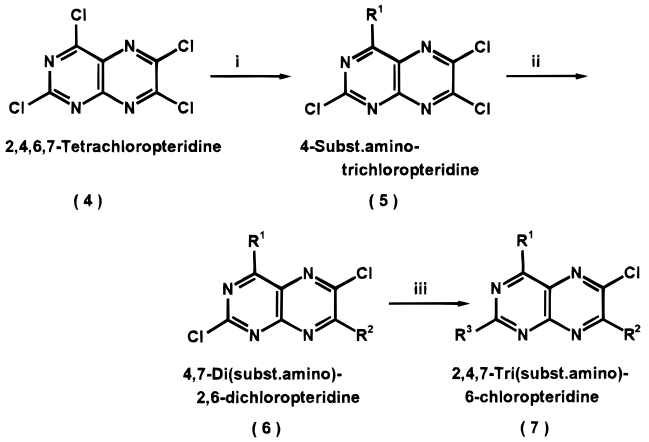
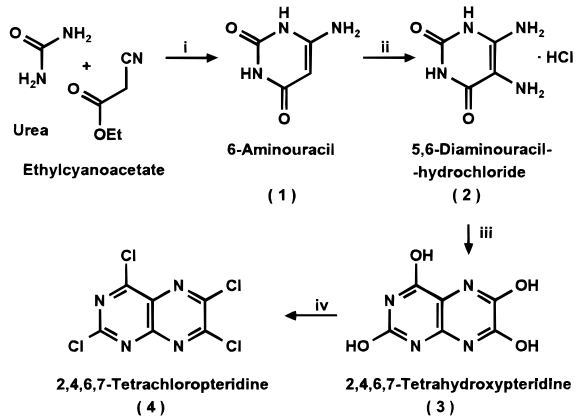
**Scheme 1**[a]




[a] Reagents and conditions: (i) pyrrolidine, 1 M $KHCO_3$, $CHCl_3$, 0 °C; (ii) benzylamine, dioxane, 25 °C; (iii) piperazine, dioxane, triethylamine, 100 °C.

**Scheme 2**[a]




[a] Reagents and conditions: (i) 1. NaOEt, EtOH, 4 h reflux, 2. HOAc; (ii) 1. $NaNO_2$, 2. $Na_2S_2O_4$, 3. HCl; (iii) oxalic acid, bath temperature 240 °C; (iv) $PCl_5/POCl_3$, reflux 16 h.

2,4,6,7-Tetrachloropteridine (**4**) is the key intermediate for the preparation of these compounds. It was prepared as shown in Scheme 2: Condensation of ethylcyanoacetate with urea gives 6-aminouracil (**1**), and subsequent nitrosation and reduction yields 5,6-diaminouracil hydrochloride (**2**).[6] Melting condensation of **2** with oxalic acid dihydrate produces 2,4,6,7-tetrahydroxypteridine (**3**).[7] Chlorination of **3** with a mixture of phosphorus pentachloride and phosphorus oxychloride affords 2,4,6,7-tetrachloropteridine (**4**) in a moderate yield.[8]

The reaction of **4** with pyrrolidine at 0 °C in aqueous hydrogen carbonate/chloroform resulted in a mixture of products as summarized in Scheme 3. Separation by column chromatography on silica gel (ethyl acetate/hexane = 3/7) provided 4-pyrrolidino-2,6,7-trichloropteridine (**5a**) (28%), as has been described in the patent literature. However, in substantial yields, 2-pyrrolidino-4,6,7-trichloropteridine (**5z**, 29%) and 4,7-bis(pyrrolidino)-2,6-dichloropteridine (**6c**, 16%) were also present in the mixture. **5z** and **6c** were separated by column chromatography on silica gel (100% dichloromethane). The reaction of the unseparated raw reaction mixture with benzylamine at room temperature provided equal amounts of **6a** and **6b**, from which 7-pyrrolidino-4-benzylamino-2,6-dichloropteridine (**6b**) could be isolated by fractionated crystallization. Hence the product mixture of the first substitution also contained 7-pyrrolidino-2,4,6-trichloropteridine in substantial amounts. As a consequence of hydrolysis during the chromatographic process, this product could, however, not be isolated. The mass spectrum revealed the signal of a molecular ion showing the presence of a pyrrolidino, two chloro, and one hydroxyl substituents (*m/e* 285 $M^+$, typical chlorine isotope distribution).

The preparation of 4,7-bis(dimethylamino)-2,6-dichloropteridine (**6f**) was accomplished by three routes (Scheme 4): (i) The reaction of **4** with an aqueous solution of dimethylamine at 0 °C in aqueous hydrogen carbonate/chloroform gave a mixture, from which 4-(dimethylamino)-2,6,7-trichloropteridine (**5e**) and **6f** could be separated by column chromatography on silica gel. A compound analogous to **5z** could not be detected. (ii) The reaction of **4** with dimethylamine in ether at 0 °C provided **6f** as the main product, and only traces of **5e** were detected by TLC. In contrast to our findings, these reaction conditions had been reported earlier to afford 6,7-bis(dimethylamino)-2,4-dichloropteridine.[9] (iii) **6f** was also prepared by reaction of **5e** with a solution of dimethylamine in THF at room temperature.

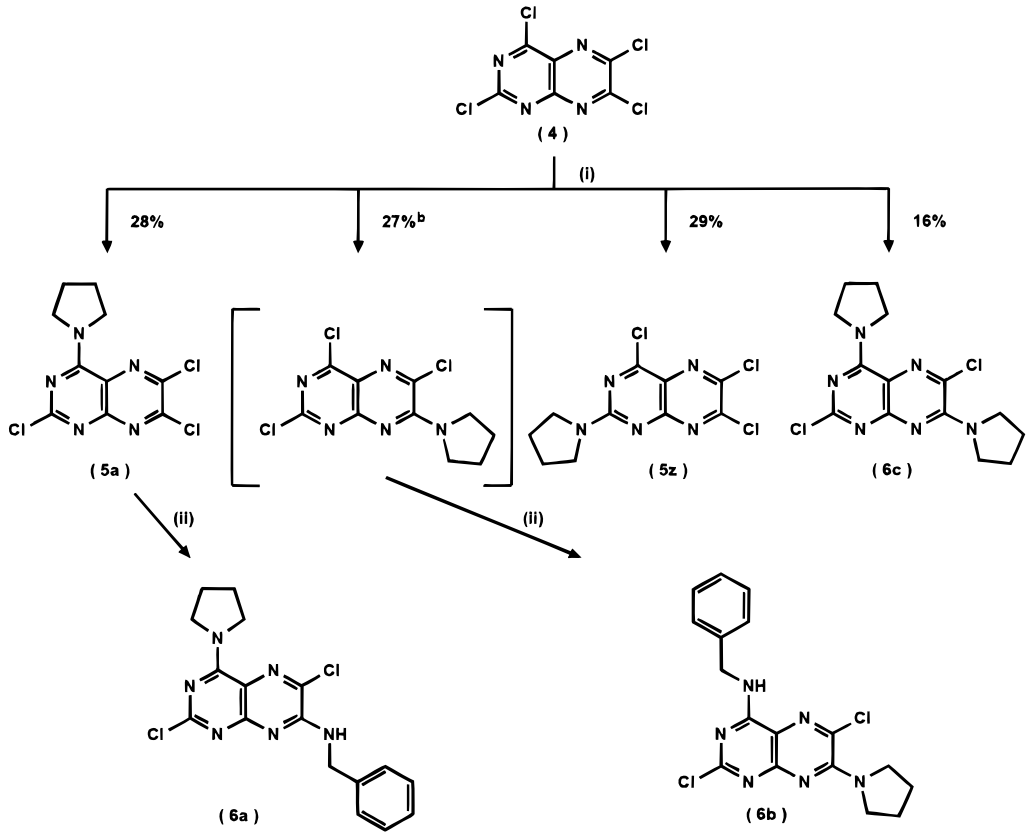
Reaction of **5a** and **5e** with benzylamine and **5a** with dimethylamine at 20 °C exclusively afforded 4,7-di(subst)amino-2,6-dichloropteridines (**6a,d,e**) as products (Scheme 1, step ii). Structures, formulas, and melting points of the intermediates are listed in Table 1.

The substitution of the third chlorine atom was accomplished by reaction at 100 °C under atmospheric pressure to provide 2,4,7-tri(subst)amino-6-chloropteridines (**7 a**−**l**) (Scheme 1, step iii). The generated HCl was scavenged either by triethylamine or by adding the reacting amine in excess.

Firm proof concerning the stepwise alkylaminolysis of chloropteridines is not available from the literature,[9−12] and therefore only a putative positional order of reactivity has been ascribed to the chloro substituents toward aminolysis until now.[13]

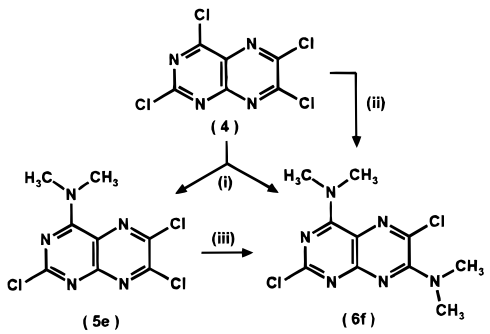
In view of the absence of hydrogen atoms at the heterocyclic ring system with exception of **7o** and also of splittings due to long-range C−H couplings in the $^{13}C$ NMR spectra, confirmation of the positional isomers was done by X-ray crystallography. The most convenient way would have been to determine the structures of **7a** and **7b**, because these structures would also provide direct proof of the configuration of **6a**, **6b**, and **5a**, respectively. We succeeded in crystallizing **7b** as a bis(hydroperchlorate) hydrate and in determining its structure. Figure 1 shows the structure of the dication of this salt. Unfortunately, **7a** failed to crystallize in a couple of solvents and by several methods. Alternatively, **6a** was crystallized from ethanol as a hemisolvate (Figure 2). Due to positional disorder of the solvent molecules in the crystal lattice and due to conformational disorder of the pyrrolidino group, the results of the structure determination are of limited quality but give an unambiguous proof of all relevant structural features. The experimental evidence from the reaction of **6b** to **7b** with its proven structure allows the conclusion that under the same reaction conditions the 2-chloro substituent reacts, resulting in the conversion of **6a** to **7a**.

**Scheme 3**[a]




[a] Reagents and conditions: (i) pyrrolidine, 1 M $KHCO_3$, $CHCl_3$, 0 °C; (ii) benzylamine, dioxane, 25 °C. [b] Calculated as difference to 100%.

**Scheme 4**[a]



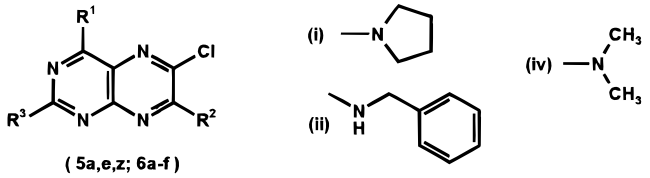

[a] Reagents and conditions: (i) dimethylamine, 40% in $H_2O$, 1 M $KHCO_3$, $CHCl_3$, 0 °C; (ii) dimethylamine, 2 M in THF, $Et_2O$, 0 °C; (iii) dimethylamine, 2 M in THF, dioxane, 25 °C.

Further insight into the reactivity toward nucleophilic attack of 2,4,6,7-tetrachloropteridine is given by the $^1$H NMR spectra, which are indicative for the strength of the bonds at position 2 and 4 of the pteridine ring formed by reacting with pyrrolidine (p$K_a$ = 11.31)[14] and dimethylamine (p$K_a$ = 10.72).[15] The $^1$H NMR spectra of **5a** and **5z** (Figure 3) show that the $C_4-N_{pyrrolidine}$ bond of **5a** has almost double-bond character. At room temperature, a rotation around this bond does not take place, as indicated by distinct triplets and quintets for the 2′- and 3′-protons (Figure 3, ii). In **5z**, slow rotation around the pteridine−substituent axis is allowed at room temperature, giving rise to the broad peaks in the $^1$H NMR spectrum (Figure 3, i).

Substitution with dimethylamine, a weaker nucleophile, exclusively gave **5e** and **6f**. No indication was found for the formation of 2-(dimethylamino)-4,6,7-trichloropteridine. The $^1$H NMR spectrum of **5e** (Figure 4, i), in analogy to **5a**, shows two sharp singlets for the methyl protons, indicating steric fixation of the substituent in 4-position. The introduction of the second dimethylamino group in 7-position causes an attenuation of the $C_4-N_{pyrrolidine}$ bond strength. The $^1$H NMR spectrum of **6f** (Figure 4, ii) shows a very broad signal for the methyl protons of the substituent in 4-position and one sharp singlet for the methyl protons of the substituent in 7-position, indicative for slow and fast rotation around the pteridine−substituent axis. The substitution of the chlorine atom in 7-position of **5a** by benzylamine is expected to reduce the $\pi$-electron deficiency of the pteridine system, too. Remarkably, however, the pattern and shape of the signals of the pyrrolidine protons in the $^1$H NMR spectrum of **6a** were unchanged, although a slight upfield shift became apparent (Figure 5, i). This may be reconciled with a smaller p$K_a$ value of benzylamine (p$K_a$ = 9.37).[15] Replacement of the chloro substituent in 2-positon by a third amino substituent (**7a**) has a stronger impact on the electron deficiency in the pyrimidine part of the molecule. This results in attenuation of the $C_4-N_{pyrrolidine}$ bond strength, enabling the pyrrolidine substituent to rotate, as indicated by the broad peaks at 1.90, 3.73, and 3.95 ppm in the $^1$H NMR spectrum (Figure 5, ii).

The isomeric dimethylamino compounds **7m** and **7n** were prepared by the reaction of gaseous ammonia with a suspension of **6a** in dimethylformamide in a sealed

**Table 1.** Structure, Formulas, and Melting Points of Di- and Trichloropteridines




( 5a,e,z; 6a-f )

| compd | $R^1$ | $R^2$ | $R^3$ | formula | analyses[a] | mp (°C) |
|---|---|---|---|---|---|---|
| **5a** | pyrrolidino | Cl | Cl | $C_{10}H_8Cl_3N_5$ | C, H, N | 245 |
| **5e** | dimethylamino | Cl | Cl | $C_8H_6Cl_3N_5$ | C, H, N | 225 |
| **5z** | Cl | Cl | pyrrolidino | $C_{10}H_8Cl_3N_5$ | C, H, N | 182 |
| **6a** | pyrrolidino | benzylamino | Cl | $C_{17}H_{16}Cl_2N_6$ | C, H, N | 152 |
| **6b** | benzylamino | pyrrolidino | Cl | $C_{17}H_{16}Cl_2N_6$ | C, H, N | 170 |
| **6c** | pyrrolidino | pyrrolidino | Cl | $C_{14}H_{16}Cl_2N_6$ | C, H, N | 169−170 |
| **6d** | pyrrolidino | dimethylamino | Cl | $C_{12}H_{14}Cl_2N_6$ | C, H, N | 239 |
| **6e** | dimethylamino | benzylamino | Cl | $C_{15}H_{14}Cl_2N_6$ | C, H, N | 225−227 |
| **6f** | dimethylamino | dimethylamino | Cl | $C_{10}H_{12}Cl_2N_6$ | C, H, N | 244 |

[a] All values were within ±0.4% of the calculated values.

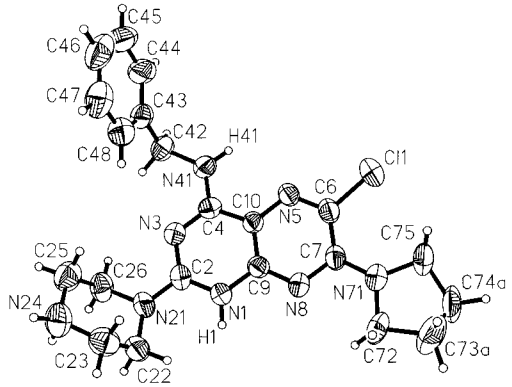



**Figure 1.** Diagram of the dication in **7b**·2$HClO_4$·$H_2O$. Displacement ellipsoids are drawn at the 50% propability level, radii of hydrogen atoms are chosen arbitrarily, and the hydrogen atom labels are omitted for clarity. Note that a 1:1 conformational disorder of the pyrrolidino group in the crystal was found. Only one of the conformations is drawn.

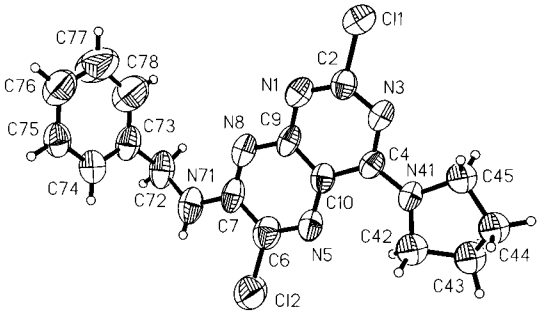



**Figure 2.** Diagram of **6a** in **6a**·0.5EtOH (displacement ellipsoids and further details as described for Figure 1). As for **7b**, a 1:1 conformational disorder of the pyrrolidino group in the crystal was found. Only one of the conformations is drawn.

glass tube at 100 °C and 12 bar and subsequent isolation by column chromatography. No indication for the formation of 2- or 6-$NH_2$ substituted compounds was found. Obviously under these conditions DMF serves as donor for dimethylamine, the formyl group of DMF presumably ending up as formamide. The structure of **7m** was established by the shifts of the C atoms in the $^{13}C$ NMR spectrum, showing the same pattern as **7a**. In contrast, the $^{13}C$ NMR spectrum of **7n** exhibits a different set of resonances for the pteridine carbon

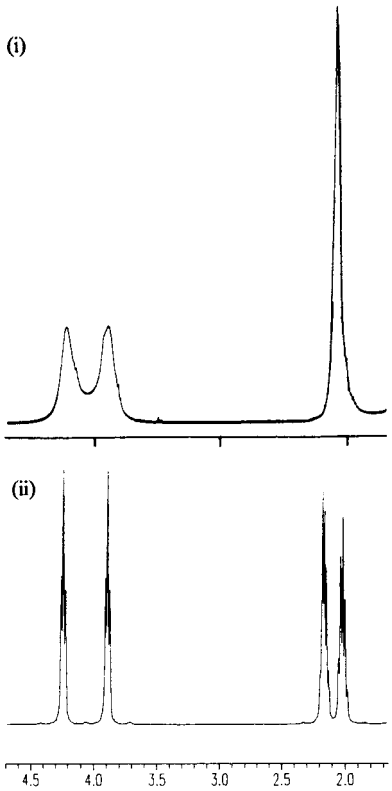



**Figure 3.** Section (1.7−4.6 ppm) of the $^1H$ NMR spectra at 293 K of (i) **5z** and (ii) **5a** in $CDCl_3$.

atoms, indicating the presence of the dimethylamino substituent in 6-position.

The hydrogenation of **7a** in the presence of 10% palladium on carbon under atmospheric pressure afforded **7o** within 2 h at room temperature.

In summary, these results demonstrate that the reactivity of the carbon atoms in 2,4,6,7-tetrachloropteridine for nucleophilic aromatic substitution with secondary amines follows the order of C4 > C7 > C2 > C6. Using the synthetic procedure as described in the patent literature,[5] the first substitution step produces not only **5a** but also **5z** and **6c** in substantial amounts. We found that chromatographic isolation of these products is mandatory for the consecutive synthesis of alkylamino substituted (chloro)pteridines free of positional isomers.

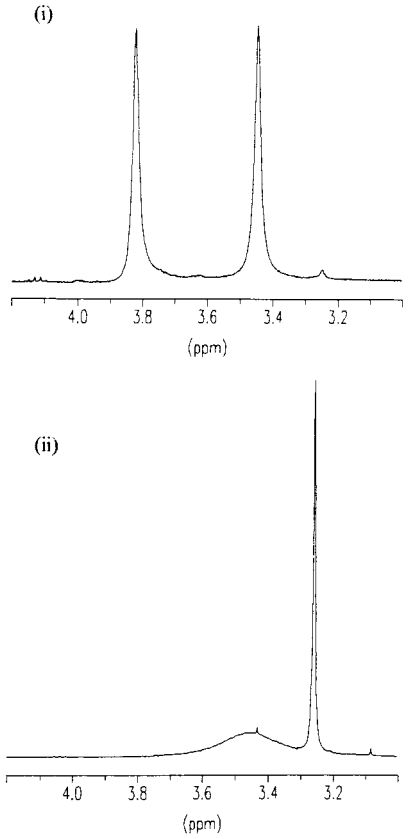



**Figure 4.** Section (3.0−4.2 ppm) of the $^1$H NMR spectra at 293 K of (i) **5e** in $CDCl_3$ and (ii) **6f** in $CDCl_3$.

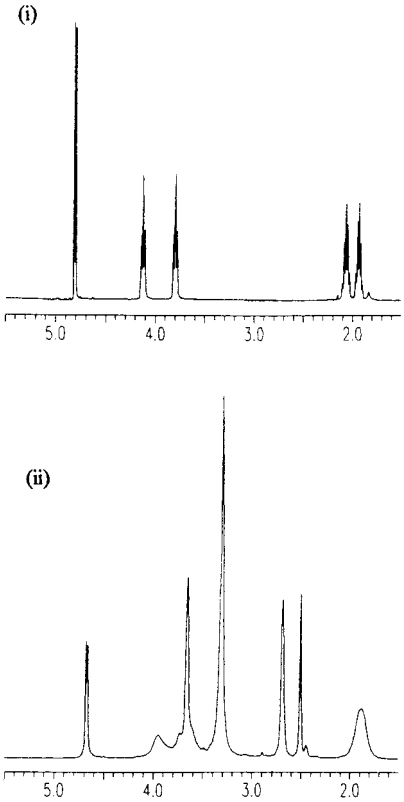



**Figure 5.** Section (1.5−5.5 ppm) of the $^1$H NMR spectra at 293 K of (i) **6a** in $CDCl_3$ and (ii) **7a** in DMSO-$d_6$.

## Biology

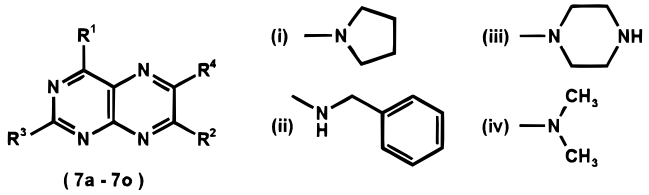
Compounds listed in Table 2 were tested for their ability to inhibit cAMP hydrolysis of purified PDE4 protein isolated from the cytosol of solid tumor tissue of the human large cell lung tumor xenograft LXFL529, grown in nude mice. The isolated protein was cAMP-specific, showing no significant cGMP hydrolysis at a nucleotide concentration of 1 $\mu$M, and exhibited high sensitivity to the selective PDE4 inhibitor rolipram ($IC_{50} = 0.1 \pm 0.06$ $\mu$M; $n = 3$), characteristic for isoenzymes of the PDE4 family. No significant changes of the cAMP hydrolysis rate were observed in the presence of cGMP or $Ca^{2+}$/calmodulin, thus proving the absence of a contamination with PDE1, PDE2, or PDE3 isoenzymes. SDS−PAGE and subsequent silver staining of the isolated protein revealed only one band at approximately 93 kDa. Western blotting with a monoclonal antibody for the PDE4D subfamily showed an immunoreactive band at the same molecular weight (data not shown). Therefore, the purified protein belongs to the PDE4D subfamily. A member of the PDE4D subfamily with the respective molecular weight has been reported as PDE4D3.[16,17]

## Results and Discussion

**7a** is a potent inhibitor of PDE4 isolated from solid tumors of the human large cell lung tumor xenograft LXFL529. Concomitantly, growth inhibition of the respective cell line is induced in the low micromolar range (Table 2). However, there is a discrepancy between the potency of the compound to inhibit isolated PDE4 and the growth inhibitory concentrations in the SRB assay (Table 2). The $IC_{50}$ values for growth inhibition in cell culture exceed the $IC_{50}$ values for PDE4 inhibition approximately by a factor of 150. This leaves the question whether PDE4 is the only relevant target for the growth inhibitory effects of **7a**. In previous studies we have shown in different tumor cell models that treatment of tumor cells with **7a** efficiently inhibits the intracellular PDE4 activity, resulting in a dose-dependent increase of the intracellular cAMP level and in growth inhibition with $IC_{50}$ values in the same order of magnitude.[2,3] Thus, the potency of the compound to inhibit isolated PDE4 from tumor tissue is different from its potential to inhibit intracellular PDE4 activity as measured by incubation of tumor cells. However, comparison of PDE4 isolated from solid tumors of LXFL529 with PDE4 from the respective cell line reveals no difference in sensitivity to **7a** (data not shown). The discrepancy between inhibition of isolated PDE4 and growth inhibition of tumor cells might be ascribed to subcellular localization of the drug which we found to concentrate to a great extent in cellular compartments other than the cytosol. It is, however, the cytosol where more than 80% of total PDE4 of LXFL529L cells is localized.[18] Moreover, cellular targets other than PDE4 might also be affected by **7a** which might be of influence to the observed biological effects.

**Structure−Activity of Substituted Pteridines. (a) Position 4 and 7 (Figure 6).** Replacement of the benzylamino substituent in 7-position by pyrrolidine (**7c**) results in a reduction of PDE4 inhibitory properties almost by a factor of 4. A dimethylamino substituent in 7-position (**7d**) provides approximately the same attenuating effect. Concomitantly, the growth inhibitory potential of **7c** and **7d** is slightly decreased com-

**Table 2.** Structure, Formulas, Melting Points, Tumor Cell Growth Inhibition Data, and PDE4 Inhibitory Properties of Tested Compounds




( 7a - 7o )

| compd | $R^1$ | $R^2$ | $R^3$ | $R^4$ | formula[a] | mp (°C) | growth inhibition $IC_{50}$ ($\mu$M)[b] | PDE4 inhibition $IC_{50}$ ($\mu$M)[c] |
|---|---|---|---|---|---|---|---|---|
| **7a** | i | ii | iii | −Cl | $C_{21}H_{25}ClN_8$ | 192−194 | 2.3 ± 0.3 | 0.016 ± 0.005 (4) |
| **7b** | ii | i | iii | −Cl | $C_{21}H_{25}ClN_8$ | 170−172 | 4.0 ± 0.2 | 0.083 ± 0.037 (2) |
| **7c** | i | i | iii | −Cl | $C_{18}H_{25}ClN_8 \cdot 0.5H_2O$ | 194 | 3.1 ± 0.1 | 0.059 ± 0.005 (2) |
| **7d** | i | iv | iii | −Cl | $C_{16}H_{23}ClN_8$ | 146−148 | 5.8 ± 0.4 | 0.046 ± 0.006 (3) |
| **7e** | iv | ii | iii | −Cl | $C_{19}H_{23}ClN_8 \cdot 0.5H_2O$ | 138−140 | 5.9 ± 0.6 | 0.30 ± 0.14 (2) |
| **7f** | iv | iv | iii | −Cl | $C_{14}H_{21}ClN_8 \cdot 0.5H_2O$ | 131−132 | >20 | 1.2 ± 0.5 (3) |
| **7g** | i | ii | 4-methylpiperazino | −Cl | $C_{22}H_{27}ClN_8 \cdot 0.5H_2O$[d] | 204 | 6.5 ± 0.5 | 3.1 ± 0.7 (3) |
| **7h** | i | ii | morpholino | −Cl | $C_{21}H_{24}ClN_7O \cdot 0.5H_2O$ | 186 | >20 | 2.5 ± 0.4 (3) |
| **7i** | i | ii | 4-acetylpiperazino | −Cl | $C_{23}H_{27}ClN_8O$ | 146−148 | 13.0 ± 1.1 | 5.9 ± 1.1 (2) |
| **7j** | i | ii | 3-oxopiperazino | −Cl | $C_{21}H_{23}ClN_8O$[e] | >250 | 9.5 ± 2.2 | 4.2 ± 0.5 (3) |
| **7k** | i | ii | 2-aminoethylamino | −Cl | $C_{19}H_{23}ClN_8$ | 177−179 | 3.2 ± 0.4 | 3.7 ± 0.5 (3) |
| **7l** | i | ii | 2-hydroxyethylamino | −Cl | $C_{19}H_{22}ClN_7O$ | 198 | 15.2 ± 1.4 | 2.8 ± 0.1 (3) |
| **7m** | i | ii | iv | −Cl | $C_{19}H_{22}ClN_7$ | 196 | 11.8 ± 0.8 | 1.8 ± 1.2 (2) |
| **7n** | i | ii | −Cl | iv | $C_{19}H_{22}ClN_7$ | 187 | >20 | 0.65 ± 0.05 (2) |
| **7o** | i | ii | iii | −H | $C_{21}H_{26}N_8$ | 218 | 7.3 ± 0.7 | 0.36 ± 0.09 (3) |

[a] Analyses for C, H, and N were within ±0.4% of the expected values for the formula unless otherwise noted. [b] Growth inhibition of LXFL529L cells was determined using the sulforhodamine B assay, incubation time 3 d. $IC_{50}$ values were calculated as survival of treated cells over control cells × 100 [T/C %]; values are given as mean ± SEM of two separate assays, each done in quadruplicate. [c] PDE4 was isolated from solid tumor tissue of the human large cell lung tumor xenograft LXFL529, grown in nude mice. Inhibition of cAMP hydrolysis was determined in the presence of 1 $\mu$M cAMP according to the method of Pöch.[20] $IC_{50}$ values were determined from the logarithmic concentration−inhibition curve (at least three points). Values are given as mean ± SEM, number of separate experiments in parentheses, each done in triplicate. [d] C: calcd, 59.0; found, 59.5. [e] N: calcd, 25.5; found, 24.9.

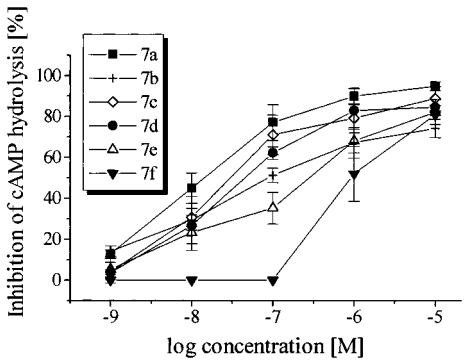



**Figure 6.** Inhibition of PDE4 activity isolated from solid tumor tissue of LXFL529 by **7a** and derivatives with modifications in 4- and/or 7-position of the pteridine ring system. Inhibition of cAMP hydrolysis was determined in the presence of 1 $\mu$M cAMP according to the method of Pöch.[20] Values are given as mean ± SD of at least two separate experiments, each done in triplicate.

pared to that of **7a**. Interchange of substituents at positions 4 and 7 of **7a** induces a 5-fold decrease in PDE4 inhibition (**7b**). The comparison of **7b** and **7c** shows that exchange of the pyrrolidine residue in 4-position versus exchange of an aromatic group has no significant impact on PDE4 inhibitory activity. Derivatives **7b**, **7c**, and **7d** are similar in their PDE4 inhibitory properties and are somewhat less potent compared to **7a**.

Replacement of pyrrolidine in 4-position by dimethylamine (**7e**) results in a stronger attenuation of PDE4 inhibition. **7e** is almost 20-fold less inhibitory toward PDE4 than **7a** and about 6-fold less inhibitory than **7d**. Both compounds induce, however, cell growth inhibition to the same extent. When both positions 4 and 7 carry dimethylamine (**7f**), PDE4 inhibitory properties are reduced by a factor of 75, compared to **7a**. The latter modification also totally abolishes growth inhibitory effectiveness.

Since compounds **7b**−**e** are still able to induce growth inhibition, albeit less effective than **7a**, it appears that space-filling substitutents larger than dimethylamine are required for optimum activity.

**(b) Position 6.** Removal of the chloro substituent in 6-position, which might potentially be involved in H bond formation, causes a dramatic loss in PDE4 inhibition, the resulting analogue (**7o**) being about 20-fold less effective than **7a** (Figure 6). However, growth inhibitory properties are reduced only by a factor of 3 (Table 2). This suggests that **7o** might influence also other cellular targets potentially relevant for growth.

**(c) Position 2.** Modifications at position 2 of the pteridine ring system (**7g**−**m**) bring about the highest impact on PDE4 inhibition (Figures 7 and 8). The substituent in position 2 of **7a** contains a strong basic center at N4′, representing also a hydrogen bond donor. N4′ methylation removes the hydrogen bond donor (**7g**). This results in a 200-fold reduction of PDE4 inhibitory properties, compared to those of **7a**. Introduction of an oxogroup in C3′-position (**7j**) removes the basicity of N4′ but preserves H bond donor qualities, showing slightly reduced PDE4 inhibition compared to that of **7g**. Acetylation of N4′ (**7i**) eliminates both basicity of N4′ and H bond donor abilities, resulting in a further loss of PDE4 inhibitory properties, **7i** being 400-fold less potent than **7a**. Complete removal of the basic center and of H bond donor activity at position 4′ is achieved by exchange of piperazine versus dimethylamine (**7m**).

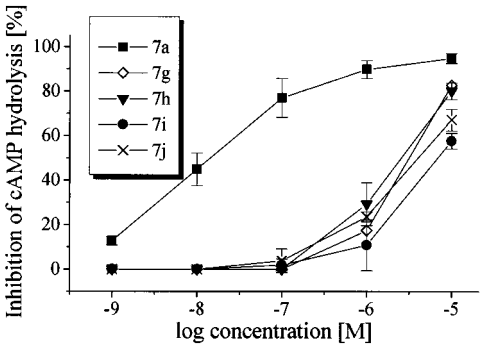



**Figure 7.** Inhibition of PDE4 activity isolated from solid tumor tissue of LXFL529 by **7a** and derivatives with modifications at the piperazino residue. Inhibition of cAMP hydrolysis was determined in the presence of 1 $\mu$M cAMP according to the method of Pöch.[20] Values are given as mean ± SD of at least two separate experiments, each done in triplicate.

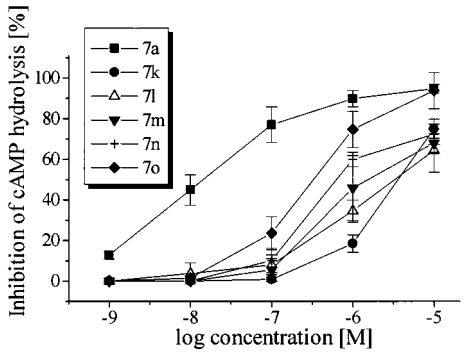



**Figure 8.** Inhibition of PDE4 activity isolated from solid tumor tissue of LXFL529 by **7a** and derivatives with modifications in 2-position of the pteridine ring system. Inhibition of cAMP hydrolysis was determined in the presence of 1 $\mu$M cAMP according to the method of Pöch.[20] Values are given as mean ± SD of at least two separate experiments, each done in triplicate.

This modification induces a strong reduction of PDE4 inhibitory properties, similar to those of **7g**. **7m** is slightly more potent than **7i**, which might be due to the different spatial requirements when interacting with the target enzyme.

The growth inhibitory efficacy of **7g** is about one-third that of **7a** on the basis of $IC_{50}$ values. With respect to PDE4 inhibition, **7g** displays about 0.5% of **7a** and 10% of **7e**, whereas the $IC_{50}$ values for growth inhibition of the latter two derivatives are very similar. Thus **7g** might affect other cellular targets.

Replacement of the H bond donor piperazine (**7a**) by the H bond acceptor morpholine (**7h**) also induces a strong loss of PDE4 inhibitory capacity, comparable to **7g**. But in contrast to **7g**, **7h** exhibits no growth inhibitory activity anymore. Apparently, the H bond donor function has a greater impact on cell growth than on PDE4 inhibition.

Substitution of the piperazine by 2-amino-ethylamine (**7k**) introduces a substituent with enhanced possibility of free rotation with the basic center in approximately the same distance to the pteridine ring system as the piperazine residue. PDE4 inhibitory property of **7k** is comparable to that of **7g**−**j**, the other derivatives with modified 4′-position. In terms of growth inhibition however, **7k** is very close to **7a**. Replacing the 2-amino-ethylamino substituent in **7k** by a 2-hydroxyethylamino group (**7l**) has no impact on PDE4 inhibition but considerable influence on tumor cell growth inhibition, **7l** exhibiting only 20% of the activity of **7k**.

Interchanging the dimethylamino substituent in 2-position and the chloro substituent in 6-position leads to a molecule (**7n**) with different spatial requirements, compared to those of **7m**. Interestingly, **7n** exhibited 3-fold higher potency to inhibit PDE4 compared to **7m** but showed no growth inhibitory properties. Since the 2-chloro substituent in **7n** shows a relatively high reactivity toward nucleophilic substitution, a reaction of **7n** with nucleophiles from the cell culture medium used in the SRB assay is believed to be the reason for the observed loss of growth inhibitory activity.

In summary, reduction of PDE4 inhibitory potency always induces a decrease of the growth inhibitory properties. The most potent compounds, **7a**−**d**, exhibit some degree of correlation between these two parameters. Regarding the derivates **7e**−**o**, which are less potent with respect to PDE4 inhibition by one or 2 orders of magnitude when compared to **7a**, the results of the SRB assay do not reflect variations in PDE4 inhibitory properties. In addition to potential differences in cellular uptake and distribution, some of the molecular modifications apparently inflict on cellular targets other than PDE4, and this might result in differential influence on growth.

## Conclusion

In this study, we have elucidated the order of reactivity of pteridine C atoms toward secondary amines and have established a synthetic route that allows the preparation of alkylamino-substituted pteridines free of positional isomers. The impact of structural elements necessary for PDE4 inhibition has been investigated. Changes in 7-position (**7c**,**d**) had minor impact on the inhibitory properties, compared to modifications in the other pteridine ring positions. Reduction of the size of the substituent in 4-position (**7e**) induces a significant reduction of PDE4 inhibition. Exchange of the chloro substituent versus hydrogen (**7o**) diminishes PDE4 inhibition by the factor of 20. The highest impact is found for modifications at position 2 of the pteridine ring system, where the parent compound carries a piperazino residue. Potent PDE4 inhibitors are also found to be potent inhibitors of tumor cell growth. However, with compounds exhibiting significantly diminished PDE4 inhibitory properties, no correlation between PDE4 and growth inhibition can be observed any longer. This indicates that inhibition or interaction with cellular targets different from PDE4 might become relevant for tumor cell growth inhibition displayed by such derivatives.

## Experimental Section

**Biology**. **Materials.** [$^3$H]-cAMP (specific activity 32.9 Ci/mmol) and [$^3$H]-cGMP (specific activity 8.5 Ci/mmol) were obtained from E. I. du Pont de Nemours & Co., (Inc). Column HR 10/2, Q-Sepharose FF gel suspension and gel filtration column Hiload 16/60 Superdex 200 were purchased from Pharmacia (Freiburg, Germany). Rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl]-2-pyrrolidone) was kindly provided by Schering AG (Berlin, Germany).

**Human Xenograft Tumor Tissue.** Human tumors established by serial passage in nude mice (NMRI genetic background) were used for obtaining tumor xenograft material.

The tumors have been described and characterized in detail.[19] Tumors were implanted subcutaneously into both flanks of the animals. When a median diameter of 10−15 mm was reached, the mouse-grown tumors were resected under sterile conditions and frozen in liquid nitrogen immediately.[19] The non-small cell lung tumor xenograft LXFL529 was established from a primary tumor of a 34 year old female without receiving chemotherapy prior to tumor resection. The survival time of the patient after resection was 115 days.

**Cytosol Preparation.** All procedures were performed at 4 °C. Tumor tissue was homogenized in homogenization buffer (50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 4 mM benzamidine hydrochloride, 0.5 $\mu$M trypsin inhibitor (soy beans), 0.1 mM phenylmethylsulfonyl fluoride, 1 mM $\beta$-mercaptoethanol, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 1 $\mu$M pepstatin, 1 $\mu$M leupeptin). After centrifugation (100000*g*, 1 h) the supernatant was carefully removed, pooled, and applied to anion exchange chromatography.

**Anion Exchange Chromatography.** The supernatant was loaded on a Q Sepharose column (HR 10/2, Pharmacia), equilibrated with buffer B (50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 4 mM benzamidine hydrochloride, 0.5 $\mu$M trypsin inhibitor (soy beans), 0.1 mM phenylmethylsulfonyl fluoride, 1 mM $\beta$-mercaptoethanol, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 1 $\mu$M pepstatin, 1 $\mu$M leupeptin). After loading, the column was washed with 20 mL of buffer B, flow rate 2 mL/min. PDE activity was eluted at the same flow rate with buffer A containing 1 M NaCl using a step gradient. Fractions of 5 mL were collected and assayed for PDE activity. Fractions containing rolipram-sensitive PDE activity were concentrated by ultrafiltration (Amicon chamber 8010, membrane YM3) under nitrogen to a volume of 1 mL. The filtration step was performed at 4 °C. The resulting samples were immediately applied to gel filtration.

**Gel Filtration.** The column was equilibrated with two bed volumes of buffer C (50 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.3 mM glycerol, 0.5 mM NaCl). Gel filtration was performed at a flow rate of 0.5 mL/min. Fractions of 2 mL were collected and immediately assayed for PDE activity and sensitivity to rolipram.

**Phosphodiesterase Assay.** PDE activity was measured as described previously.[2,20] Briefly, PDE-containing preparations were incubated with [$^3$H]-cAMP in buffer A, containing 50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM AMP. The final cAMP concentration in the assay was 1 $\mu$M. Incubations were routinely carried out in triplicate at 37 °C. Reaction was stopped by adding $ZnSO_4$. [$^3$H]-5′-AMP was precipitated by addition of $Ba(OH)_2$ and centrifuged at 10000*g*. Nonhydrolyzed [$^3$H]-cAMP was determinated by liquid scintillation counting of the supernatant. Calculation of PDE activity was carried out with reference to controls.

**Sulforhodamin B Assay.** Cells were cultivated in humified incubators (37 °C, 5% $CO_2$). LXFL529 cells were grown in RPMI 1640, with additional glutamine (300 mg/L), 1% penicillin/streptomycin, and 10% newborn calf serum. The cells were free from mycoplasm contamination, as tested routinely. Cells were seeded in 24-well plates and allowed to grow 24 h before treatment. The tests were performed by continuous drug incubation. Cytotoxicity was determined as described previously[21] and calculated as survival of treated cells over control cells × 100 [% T/C].

**Chemistry**. Except for dimethylamine, amines were distilled from potassium hydroxide. THF, dioxane, and ether were distilled over natrium under a dry argon atmosphere. Other solvents and reagents obtained from commercial suppliers were at least of reagent grade and were used without further purification. All reactions involving oxygen or moisture-sensitive compounds were performed under a dry argon atmosphere. All reaction mixtures and column chromatographic fractions were analyzed by thin layer chromatography on TLC plates "Alugram Sil G/$UV_{254}$" purchased from Macherey & Nagel. Column chromatography was carried out using silica gel 60, Macherey & Nagel, (0.063−0.2 mm). Melting points were determined on a Büchi melting point apparatus and are uncorrected. Elemental analyses were performed using a Perkin-Elmer 2400 CHN elemental analyzer. Unless otherwise indicated, $^1$H NMR spectra were recorded at room temperature at 400 MHZ on a Bruker AMX 400 with tetramethylsilane, DMSO ($\delta$ 2.49), or $CDCl_3$ ($\delta$ 7.26) used as internal standard. $^{13}$C NMR spectra were obtained at 100 MHZ using tetramethylsilane, $CDCl_3$ ($\delta$ 77.0), or DMSO ($\delta$ 39.5) as internal standard. *J* values are reported in hertz. Apparent multiplicities are designated as s, singlet; d, doublet; t, triplet; q, quartet; qu, quintet; m, multiplet; b, broad. Mass spectra were taken in the positive ion mode under electron impact (EI), 70 eV.

**2,4,6,7-Tetrachloropteridine (4).** This compound was prepared as described by Schöpf.[8] For most preparations, it is not necessary to sublimate the product in vacuo, as during the next reaction step a purification by column chromatography is required: $^{13}$C NMR ($CHCl_3$) $\delta$ 129.8, 149.2, 153.1, 156.1, 160.2, 165.1. Anal. ($C_6Cl_4N_4$) C, H, N.

**4-Pyrrolidino-2,6,7-trichloropteridine (5a).** A suspension of **4** (1.23 g, 4.56 mmol) in 40 mL of $CHCl_3$ was cooled to 0 °C and mixed with a solution of 912 mg of $KHCO_3$ (9.12 mmol) in 10 mL of water. A precooled solution of 325 mg of pyrrolidine (4.56 mmol) in 10 mL of $CHCl_3$ was added slowly, and the reaction mixture was stirred at 0 °C for 1 h. Water (40 mL) was added, and the organic layer was separated, dried over $Na_2SO_4$, and concentrated in vacuo to afford a yellow-brownish residue (921 mg). Column chromatography (ethyl acetate/hexane = 3/7 v/v) provided a uniform fraction of 416 mg (45.2%) with $R_f$ = 0.30 and 259 mg (28.1%) of **5a** with $R_f$ = 0.54 as pale-yellow crystals; mp 245 °C: $^1$H NMR ($CDCl_3$) $\delta$ 2.02 (qu, $^3J$ = 6.8 Hz, 2), 2.16 (qu, $^3J$ = 6.8 Hz, 2), 3.88 (t, $^3J$ = 6.9 Hz, 2), 4.24 (t, $^3J$ = 6.9 Hz, 2); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.6, 26.9, 51.1, 51.4, 124.5, 141.3, 151.4, 154.5, 157.3, 161.8; MS *m/e* 303 ($M^+$). Anal. ($C_{10}H_8Cl_3N_5$) C, H, N.

**4-Dimethylamino-2,6,7-trichloropteridine (5e)** was prepared in the same manner as **5a**. Starting with 1.5 g of **4** the reaction was performed using a 40% aqueous solution of dimethylamine to give 967 mg of raw product. Column chromatography yielded 294 mg (30.4%) of **5e**, light-yellow fine crystals; $R_f$ = 0.52 (ethyl acetate/hexane = 1/3 v/v), mp 225 °C: $^1$H NMR ($CDCl_3$) $\delta$ 3.45 (s, 3), 3.82 (s, 3); $^{13}$C NMR ($CDCl_3$) $\delta$ 41.7, 42.3, 124.5, 140.6, 151.3, 154.8, 159.1, 161.3; MS *m/e* 277 ($M^+$). Anal. ($C_8H_6Cl_3N_5$) C, H, N.

**2-Pyrrolidino-4,6,7-trichloropteridine (5z).** The fraction with $R_f$ = 0.30 from the preparation of **5a** was submitted to a further separation by column chromatography on silica gel (100% dichloromethane) to provide 264 mg (28.7%) of **5z** with $R_f$ = 0.38. The compound crystallized in bright-yellow needles; mp 182 °C: $^1$H NMR ($CDCl_3$) $\delta$ 2.08 (br, 4), 3.89 (br, 2), 4.22 (br, 2); $^{13}$C NMR ($CDCl_3$) $\delta$ 24.0, 27.3, 51.8, 124.4, 138.8, 152.6, 155.6, 158.8, 161.1; MS *m/e* 303 ($M^+$). Anal. ($C_{10}H_8Cl_3N_5$) C, H, N.

**7-Benzylamino-2,6-dichloro-4-pyrrolidinopteridine (6a).** A suspension of 964 mg (3.17 mmol) of **5a** in 25 mL of dioxane was stirred for 1 h with 488 $\mu$L (3.48 mmol) of triethylamine and 380 $\mu$L of (3.48 mmol) benzylamine at room temperature. The reaction mixture was evaporated almost to dryness and thoroughly washed with 50 mL of water, filtered, and dried over phosphorus pentoxide to afford 1083 mg (91%) of **6a**; mp 152 °C. $^1$H NMR ($CDCl_3$) $\delta$ 1.93 (qu, $^3J$ = 6.8 Hz, 2), 2.07 (qu, $^3J$ = 6.8 Hz, 2), 3.80 (t, $^3J$ = 6.8 Hz, 2), 4.12 (t, $^3J$ = 6.8 Hz, 2), 4.81 (d, $^3J$ = 5.3 Hz, 2), 6.05 (t, $^3J$ = 5.3 Hz, 1), 7.28−7.38 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.7, 26.9, 45.6, 50.2, 50.9, 117.3, 128.0, 128.1, 128.3, 128.9, 131.3, 137.2, 151.5, 156.3, 157.2, 160.3; MS *m/e* 374 ($M^+$). Anal. ($C_{17}H_{16}Cl_2N_6$) C, H, N.

**Crystal Growing of 6a.** Boiling ethanol (0.5 mL) was added to 5 mL of a solution of **6a** in ethanol, saturated at 50 °C. The solution was cooled slowly to room temperature and was kept at this temperature for 3 months under light protection. Then the mixture was stored at 4 °C until crystal growth was completed.

**Crystal Structure Determination of 6a·0.5EtOH.** $M_r$ ($C_{18.5}H_{18.5}Cl_2N_6O_{0.5}$) = 403.79, monoclinic space group *C*2/*c*, *a*

= 21.937(4) Å, $b$ = 8.893(2) Å, $c$ = 21.586(4) Å, $\beta$ = 114.72(3)°, $V$ = 3825.2(13) Å$^3$, $Z$ = 8, $D_x$ =1.402 g cm$^{-3}$, $\mu$ = 0.358 mm$^{-1}$ ($\lambda$ = 0.71073 Å), $T$ = 293 K, pale-yellow crystal of dimensions 0.5 mm × 0.3 mm × 0.2 mm in a thin walled glass capillary, 3642 reflection data were collected on an Enraf-Nonius CAD4 diffractometer. Lp corrections were applied. The structure was solved by direct methods[22] and refined (216 parameters) by full-matrix least-squares calculations on $F^2$,[23] using all but three of the most negative of 1858 unique reflections to final $R_1[F_o^2 > 2\sigma(F_o^2)]$ = 0.067, $wR_2$ = 0.198 (all data used for refinement), $w = 1/[\sigma^2(F_o^2) + (0.10P)^2 + 6.9P]$ where $P = (F_o^2 + 2F_c^2)/3$, $S$ = 1.068.[23] Largest peak and hole in the final difference map are 0.257 e/Å$^3$ and −0.395 e/Å$^2$, respectively. Anisotropic displacement parameters were refined for the atoms of the central dichloropteridine unit and the benzylamino group. An empirical extinction parameter was refined.[23] With two exceptions all hydrogen atoms were included in the refinement in calculated postions and were allowed to ride on the atom to which they are attached. The positions of the H atom at N71 and at the OH group of ethanol were taken from the difference map and refined, applying distance restraints only. Depending on the nature of the group, the isotropic displacement parameters of the H atoms were kept equal to 120%, 130%, and 150% of the equivalent isotropic displacement parameters of the parent atoms, respectively.

**4-Benzylamino-2,6-dichloro-7-pyrrolidinopteridine (6b).** A 1.35 g (5 mmol) sample of **4**, purified by sublimation in vacuo, was used as described for the preparation of **5a** to yield 1.13 g (73.5%) of a product mixture. This mixture (3.71 mmol, calculated as pyrrolidinotrichloropteridine) was suspended in 20 mL of dioxane, and benzylamine (890 $\mu$L, 874 mg, 8.16 mmol) was added at 20 °C. The reaction mixture was stirred for 1 h, the solvent was removed in vacuo, and the yellow residue was washed with 50 mL of water. Filtration yielded 1.2 g (3.2 mmol, 86.3%) of raw product. Fractionated crystallization in ethanol yielded 330 mg (23.7%) of **6b**, which crystallized first; yellow platelets, mp 170 °C: $^1$H NMR (DMSO-$d_6$) $\delta$ 1.93 (br, 4), 3.79 (br, 4), 4.64 (d, $^3J$ = 6.3 Hz, 2), 7.23−7.35 (m, 5), 9.11 (t, $^3J$ = 6.3 Hz, 1); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 25.0, 43.6, 50.4, 115.1, 126.8, 127.4, 128.2, 132.9, 138.6, 152.5, 153.2, 159.3, 159.6; MS *m/e* 374 (M$^+$). Anal. ($C_{17}H_{16}Cl_2N_6$) C, H, N.

**4,7-Bis-(pyrrrolidino)-2,6-dichloropteridine (6c).** The second component of the separation of **5z** by column chromatography on silica gel (100% dichloromethane) with $R_f$ = 0.08 was **6c**, which was eluted by adding 10% methanol to the dichloromethane. On concentrating the solution, the compound crystallized in yellow plates; mp 169−170 °C, yield 150 mg (16.3%): $^1$H NMR ($CDCl_3$) $\delta$ 1.94 (qu, $^3J$ = 6.9 Hz, 2), 1.98−2.01(m, 4H), 2.08 (qu, $^3J$ = 6.9 Hz, 2), 3.80 (t, $^3J$ = 6.9 Hz, 2), 3.92 (br, 4), 4.14 (t, $^3J$ = 6.9 Hz, 2); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.6, 25.5, 26.8, 49.9, 50.5, 50.7, 116.9, 130.1, 151.5, 155.2, 157.0, 160.1; MS *m/e* 338 (M$^+$). Anal. ($C_{14}H_{16}Cl_2N_6$) C, H, N.

**2,6-Dichloro-7-(dimethylamino)-4-pyrrolidinopteridine (6d).** The product was analogously prepared as described for **6a**, starting with 132 mg (0.43 mmol) of **5a** and using 66.4 $\mu$L (0.47 mmol) of triethylamine. The 1.1-fold molar ratio of dimethylamine was added as 2 M solution in THF (238 $\mu$L); yield 130 mg (95.9%), yellow platelets, mp 239 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.94 (qu, $^3J$ = 6.8 Hz, 2), 2.07 (qu, $^3J$ = 6.8 Hz, 2), 3.31 (s, 6), 3.79 (t, $^3J$ = 6.8 Hz, 2), 4.14 (t, $^3J$ = 6.8 Hz, 2); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.7, 26.8, 41.2, 50.0, 50.8, 118.1, 131.3, 154.6, 154.7, 157.0, 160.3; MS *m/e* 312 (M$^+$). Anal. ($C_{12}H_{14}Cl_2N_6$) C, H, N.

**7-Benzylamino-2,6-dichloro-4-dimethylaminopteridine (6e).** Preparation as described for **6a**, starting with 160 mg (0.574 mmol) of **5e**, adding 88 $\mu$L (0.632 mmol) of triethylamine and 69 $\mu$L (0.632 mmol) of benzylamine; yield 190 mg (94.7%), recrystallization from ethanol, mp 225−227 °C: $^1$H NMR ($CDCl_3$) $\delta$ 3.52 (br, 6), 4.80 (d, $^3J$ = 5.5 Hz, 2), 6.06 (s, 1), 7.28−7.35 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 41.4, 45.5, 117.3, 127.9, 128.1, 128.8, 130.7, 137.0, 151.3, 156.6, 158.9, 159.8; MS *m/e* 348 (M$^+$). Anal. ($C_{15}H_{14}Cl_2N_6$) C, H, N.

**4,7-Bis(dimethylamino)-2,6-dichloropteridine (6f).** This compound was prepared by three ways: (a) during the preparation of **5e**, 416 mg (43.0%) of a second component (**6f**) could be isolated from the column with $R_f$ = 0.25, which crystallized upon evaporating the solvent; (b) the preparation analogous to **6d** where the reaction of **5e** (152 mg, 0.55 mmol) with 2 M THF solution of dimethylamine (300 $\mu$L, 0.6 mmol) in the presence of triethylamine (83 $\mu$L, 0.6 mmol) at room temperature yielded 142 mg (91%) of **6f**; and (c) the preparation according to Schenker[9] where a sample of **4** (270 mg, 1 mmol) was dissolved in 30 mL of ether and cooled to 0 °C. Precooled 2 M THF solution of dimethylamine (5 mL, 10 mmol) was added by a syringe, and the mixture was stirred for 1 h. The reaction mixture was evaporated to dryness and treated with 25 mL of water to remove dimethylamine hydrochloride. The yellow residue (225 mg, 78.4%) contained traces of **5e**, as seen by TLC, and was chromatographed on a column (silica gel, ethyl acetate/hexane = 1:2 v/v) to afford 190 mg (66.2%) of **6f**; greenish yellow needles, mp 244 °C: $^1$H NMR ($CDCl_3$) 298 K $\delta$ 3.33 (s, 6), 3.53 (br, 6); 223 K $\delta$ 3.35 (s, 6), 3.38 (s, 3), 3.73 (s, 3); $^{13}$C NMR ($CDCl_3$) $\delta$ 41.10, 41.14, 118.0, 130.5, 154.3, 155.0, 158.8, 159.9; MS *m/e* 286 (M$^+$). Anal. ($C_{10}H_{12}Cl_2N_6$) C, H, N.

**7-Benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine (7a).** A sample of 1 g (2.66 mmol) of **6a** and 920 mg (10.64 mmol) of finely ground piperazine in 30 mL of dioxane were heated under reflux until complete disappearance of educt, as monitored by TLC (ethyl acetate/hexane = 1/1 v/v). After the solvent was removed in vacuo, the residue was washed with 50 mL of water and the slurry spun down at 5000 rpm. The pellet was dissolved in 0.1 N HCl, filtered, and precipitated by 2% $NH_3$ solution. The solid was collected by filtration and washed. Drying over KOH provided 983 mg (87%) of a light-yellow solid; mp 192−194 °C: $^1$H NMR (DMSO-$d_6$) $\delta$ 1.90 (br, 4), 2.69 (t, $^3J$ = 4.4 Hz, 4), 3.62 (br, 1), 3.66 (t, $^3J$ = 4.4 Hz, 4), 3.73 (br, 2), 3.95 (br, 2), 4.67 (d, $^3J$ = 6.1 Hz, 2), 7.23−7.32 (m, 5), 7.98 (t, $^3J$ = 6.1 Hz, 1); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 43.4, 44.6, 45.6, 113.8, 125.5, 126.6, 126.7, 128.2, 139.2, 151.5, 156.3, 156.7, 159.5; MS *m/e* 424 (M$^+$). Anal. ($C_{21}H_{25}ClN_8$) C, H, N.

**4-Benzylamino-6-chloro-2-piperazino-7-pyrrolidinopteridine (7b).** The product mixture from the preparation of **6b** (448 mg, 1.2 mmol) and finely ground piperazine (460 mg, 5.3 mmol) were suspended in 15 mL of dioxane and refluxed for 1 h. The solvent was removed in vacuo, and the residue was washed with water and dried to afford 460 mg (90.4%) of a yellow solid. Repeated column chromatography (column length 80 cm, silica gel, 2-propanol/triethylamine = 9/1 v/v) provided 24.5% of **7a** ($R_f$ = 0.30) and 25% of **7b** ($R_f$ = 0.17), yellow solid, mp 170−172 °C: $^1$H NMR (DMSO-$d_6$) $\delta$ 1.88 (br, 4), 2.69 (br, 4), 3.70 (br, 9), 4.67 (d, $^3J$ = 5.6 Hz, 2), 7.21−7.37 (m, 5), 8.32 (br s, 1); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 25.1, 43.5, 44.6, 45.4, 50.1, 105.4, 112.9, 126.6, 127.5, 128.1, 139.9, 152.7, 154.2, 158.2, 160.2; MS *m/e* 424 (M$^+$). Anal. ($C_{21}H_{25}ClN_8$) C, H, N.

**Crystal Growing of 7b.** A sample of **7b** (20.8 mg, 0.049 mmol) was suspended in 4 mL of 0.1 N $HClO_4$. Ethanol (5 mL) was added, and the mixture was warmed to 50 °C, until complete solution had taken place. The solution was cooled slowly to room temperature and was kept at this temperature for 3 months under light protection. Then the mixture was stored at 4 °C until crystal growth was completed.

**Crystal Structure Determination of 7b·2HClO$_4$·H$_2$O.** $M_r$ ($C_{21}H_{23}Cl_3N_8O_9$) = 637.82, orthorhombic space group $P2_12_12_1$, $a$ = 8.645(2) Å, $b$ = 12.338(2) Å, $c$ = 26.471(5) Å, $V$ = 2823.5(10) Å$^3$, $Z$ = 4, $D_x$ = 1.500 g cm$^{-3}$, $\mu$ = 0.388 mm$^{-1}$ ($\lambda$ = 0.71073 Å), $T$ = 293 K, colorless crystal of dimensions 0.3 mm × 0.25 mm × 0.2 mm in a thin walled glass capillary, 20517 reflection ($\Theta_{max}$ = 25.05°), data were collected on a STOE IPDS diffractometer. The structure was solved by direct methods[22] and refined (438 parameters) by full-matrix least-squares calculations on $F^2$,[23] using all 4823 unique reflections to final $R_1[F_o^2 > 2\sigma(F_o^2)]$ = 0.0515, $wR_2$ = 0.1160, $w = 1/[\sigma^2(F_o^2) + (0.033P)^2 + 0.7P]$ where $P = (F_o^2 + 2F_c^2)/3$, $S$ = 1.083.[23] The

absolute structure has been determined by refinement of a Flack parameter (−0.04(9)).[24] Largest peak and hole in the final difference map are 0.383 e/Å$^3$ and −0.261 e/Å$^2$, respectively. Anisotropic displacement parameters were refined for all non-hydrogen atoms. All $CH_2$ hydrogen atoms were included in the refinement in calculated postions and were allowed to ride on the atom to which they are attached. The hydrogen atom positions belonging to $NH_2$ and NH groups were refined free with a restrained N distance. Depending on the nature of the group, the isotropic displacement parameters of the H atoms were kept equal to 120% and 130% of the equivalent isotropic displacement parameters of the parent atoms, respectively.

**4,7-Bis(pyrrolidino)-6-chloro-2-piperazinopteridine (7c).** A sample of 394 mg (1.16 mmol) of **6c** was suspended in 20 mL of dioxane. Ground piperazine (400 mg, 4.64 mmol) was added, and the mixture was heated under reflux for 1 h. The solvent was removed in vacuo. The residue was thoroughly washed with 30 mL of water, filtered, and dried in vacuo over KOH, yield 90.2%. Column chromatography (silica gel, ethanol + 2.5% triethylamine) provided 271 mg (60.1%) of **7c**. For analytical specimen, the product was dissolved in 0.1 N HCl and neutralized with 2% $NH_3$. The precipitate was filtered and dried over KOH; yellow solid, mp 194 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.90−1.96 (m, 9), 2.88 (t, $^3J$ = 5.1 Hz, 4), 3.71 (br, 2), 3.84−3.86 (m, 8), 4.08 (br, 2); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.7, 25.6, 27.0, 45.1, 46.2, 49.1, 50.1, 115.1, 125.2, 152.1, 156.3, 156.8, 160.5; MS *m/e* 388 ($M^+$). Anal. ($C_{18}H_{25}ClN_8$·0.5 $H_2O$) C, H, N.

**6-Chloro-7-(dimethylamino)-2-piperazino-4-pyrrolidinopteridine (7d).** See **7c**. Preparation started with 125 mg (0.4 mmol) of **6d** in 15 mL of dioxane, and the yield after column chromatography was 77 mg (53.0%) of **7d**; yellow solid, mp 146−148 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.70 (br, 1), 1.91 (br, 2), 2.01 (br, 2), 2.89 (t, $^3J$ = 5.1 Hz, 4), 3.20 (s, 6), 3.72 (br, 2), 3.87 (t, $^3J$ = 5.1 Hz, 4), 4.11 (br, 2); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.8, 27.0, 41.0, 45.2, 46.3, 49.2, 50.2, 116.5, 127.2, 155.6, 156.0, 156.9, 160.5; MS *m/e* 362 ($M^+$). Anal. ($C_{16}H_{23}ClN_8$) C, H, N.

**7-Benzylamino-6-chloro-4-(dimethylamino)-2-piperazinopteridine (7e).** See **7c**. Preparation started with 176 mg (0.504 mol) of **6e** in 15 mL of dioxane, and the yield after column chromatography was 102 mg (50.7%) of **7e**; yellow solid, mp 138−140 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.78 (br, 1), 2.91 (t, $^3J$ = 5.0 Hz, 4), 3.45 (s, 6), 3.88 (t, $^3J$ = 5.0 Hz, 4), 4.81 (d, $^3J$ = 5.2 Hz, 2), 5.66 (t, $^3J$ = 5.2 Hz, 1), 7.28−7.38 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 41.0, 45.1, 45.5, 46.2, 114.9, 125.2, 127.7, 128.1, 128.8, 137.7, 151.2, 157.7, 158.9, 159.9; MS *m/e* 398 ($M^+$). Anal. ($C_{19}H_{23}ClN_8$·0.5 $H_2O$) C, H, N.

**4,7-Bis(dimethylamino)-6-chloro-2-piperazinopteridine (7f).** See **7c**. Preparation started with 240 mg (0.836 mmol) of **6f** in 20 mL of dioxane, and the yield after column chromatography was 196 mg (69.6%) of **7f**; yellow solid, mp 131−132 °C: $^1$H NMR ($CDCl_3$) $\delta$ 2.12 (br, 1), 2.89 (t, $^3J$ = 5.0 Hz, 4), 3.20 (s, 6), 3.42 (s, 6), 3.85 (t, $^3J$ = 5.0 Hz, 4); $^{13}$C NMR ($CDCl_3$) $\delta$ 40.8, 40.9, 45.1, 46.1, 116.3, 126.3, 155.2, 156.2, 158.8, 160.0; MS *m/e* 336 ($M^+$). Anal. ($C_{14}H_{21}ClN_8$·0.5 $H_2O$) C, H, N.

**7-Benzylamino-6-chloro-2-(4-methylpiperazino)-4-pyrrolidinopteridine (7 g).** **6a** (600 mg, 1.6 mmol) and 1-methylpiperazine (640 mg, 6.4 mmol) in 30 mL of dioxane were heated under reflux until complete disappearance of educt, as monitored by TLC (ethyl acetate/hexane = 1/1 v/v). The solvent was removed in vacuo, the residue was washed two times with 40 mL of water, filtered, and dried over KOH; yellow solid, yield 626 mg (89.1%), mp 204 °C: $^1$H NMR (DMSO-$d_6$) $\delta$ 1.90 (br, 4), 2.19 (s, 3), 2.30 (t, $^3J$ = 4.9 Hz, 4), 3.67 (br, 2), 3.72 (t, $^3J$ = 4.4 Hz, 4), 3.97 (br, 2), 4.67 (d, $^3J$ = 6.4 Hz, 2), 7.22−7.32 (m, 5), 8.01 (t, $^3J$ = 6.4 Hz, 1); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 23.1, 26.4, 43.4, 43.5, 45.8, 49.0, 49.9, 54.6, 113.8, 125.5, 126.7, 126.8, 128.3, 139.2, 151.5, 156.4, 156.8, 159.6; MS *m/e* 438 ($M^+$). Anal. ($C_{22}H_{27}ClN_8$·0.5 $H_2O$) H, N. C: calcd, 59.0%; found, 59.5%.

**7-Benzylamino-6-chloro-2-morpholino-4-pyrrolidinopteridine (7h).** See **7g**. Preparation started with 173 mg (0.461 mmol) of **6a** in 12 mL of dioxane, and morpholine (160 $\mu$L, 1.84 mmol) was added; yield 175 mg (89.1%) of **7h**, yellow solid, mp 186 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.94 (br, 2), 2.01 (br, 2), 3.70 (br, 2), 3.74 (t, $^3J$ = 4.7 Hz, 4), 3.91 (t, $^3J$ = 4.7 Hz, 4), 4.11 (br, 2), 4.81 (d, $^3J$ = 5.2 Hz, 2), 5.66 (s, 1), 7.30−7.37 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.6, 27.0, 44.5, 45.6, 49.4, 50.2, 67.1, 115.1, 126.1, 127.7, 128.0, 128.8, 137.8, 151.4, 157.0, 157.4, 160.4; MS *m/e* 425 ($M^+$). Anal. ($C_{21}H_{24}ClN_7O$·0.5 $H_2O$) C, H, N.

**2-(4-Acetylpiperazino)-7-benzylamino-6-chloro-4-pyrrolidinopteridine (7i).** To a suspension of **7a** (228 mg, 0.536 mmol) in 10 mL of dioxane was added triethylamine (80 $\mu$L, 0.574 mmol). A solution of 101 $\mu$L (1.07 mmol) of acetic anhydride in 5 mL of dioxane was added slowly, and the mixture was stirred until no educt could be monitored by TLC. The reaction mixture was evaporated to dryness, washed with water, and dried. The yellow residue was purified by column chromatography (silica gel, ethanol/ethyl acetate = 1/1 v/v, $R_f$ = 0.58) to yield 186 mg (74.3%) of fine light-yellow needles; mp 146−148 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.93 (br, 2), 2.02 (br, 2), 2.13 (s, 3), 3.50 (t, $^3J$ = 5.2 Hz, 2), 3.67 (t, $^3J$ = 5.2 Hz, 2), 3.73 (br, 2), 3.90 (t, $^3J$ = 5.2 Hz, 2), 3.96 (t, $^3J$ = 5.2 Hz, 2), 4.11 (br, 2), 4.81 (d, $^3J$ = 5.3 Hz, 2), 5.71 (t, $^3J$ = 5.3 Hz, 1), 7.29−7.36 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 21.4, 23.7, 26.9, 41.5, 43.7, 44.0, 45.6, 46.4, 49.4, 50.2, 115.0, 126.3, 127.7, 128.0, 128.8, 137.7, 151.4, 157.0, 157.2, 160.1, 169.1; MS *m/e* 466 ($M^+$). Anal. ($C_{23}H_{27}ClN_8O$) C, H, N.

**2-Oxopiperazine.** The compound was synthesized according to the method of Aspinall.[25] Yield of the crude product was 57.8%. Recrystallization afforded hygroscopical colorless platelets; mp 132−133 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.94 (br, 1), 3.02 (t, $^3J$ = 5.4 Hz, 2), 3.36 (dt, $^3J$ = 5.4 Hz, $^4J$ = 2.3 Hz, 2), 3.51 (s, 2), 7.49 (br, 1); $^{13}$C NMR ($CDCl_3$) $\delta$ 42.2, 42.8, 49.7, 170.6. Anal. ($C_4H_8N_2O$) C, H, N.

**7-Benzylamino-6-chloro-2-(3-oxopiperazino)-4-pyrrolidinopteridine (7j).** To a suspension of **6a** (375 mg, 1 mmol) in 15 mL of dioxane was added 2-oxopiperazine (400 mg, 4 mmol). The mixture was stirred at 80 °C until no educt could be monitored by TLC (1:1 ethyl acetate/ hexane v/v). The solvent was removed in vacuo, and the residue was washed well with water and purified by column chromatography (silica gel, 5:1 ethyl acetate/ethanol v/v); pale-yellow solid, mp >250 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.92 (br, 2), 2.05 (br, 2), 3.47 (m, 2), 3.76 (br, 2), 4.13 (br, 2), 4.18 (t, $^3J$ = 5.3 Hz, 2), 4.54 (s, 2), 4.82 (d, $^3J$ = 5.2 Hz, 2), 5.71 (t, $^3J$ = 5.2 Hz, 1), 6.27 (s, 1), 7.31−7.39 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.7, 27.0, 40.6, 41.5, 45.7, 48.4, 49.6, 50.4, 115.0, 126.7, 127.8, 128.1, 128.9, 137.6, 151.4, 156.6, 156.8, 160.5, 168.8; MS *m/e* 438 ($M^+$). Anal. ($C_{21}H_{23}ClN_8O$) C, H. N: calcd, 25.5%; found, 24.9%.

**2-(2-Aminoethylamino)-7-benzylamino-6-chloro-4-pyrrolidinopteridine (7k).** See **7g**. Preparation started with 139 mg (0.37 mmol) of **6a** in 10 mL of dioxane. Ethylenediamine (200 $\mu$L, 3 mmol) was added, and the mixture was refluxed for 3 h; yield 120 mg (81.3%), bright-yellow solid, mp 177−179 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.89 (br, 4), 2.00 (br, 2), 2.90 (t, $^3J$ = 5.6 Hz, 2), 3.53 (br, 2), 3.69 (br, 2), 4.09 (br, 2), 4.82 (d, $^3J$ = 4.6 Hz, 2), 5.44 (br, 1), 5.70 (br, 1), 7.28−7.34 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.7, 26.9, 41.9, 44.3, 45.3, 49.4, 50.3, 115.3, 125.7, 127.6, 127.9, 128.7, 137.8, 151.2, 157.2, 157.3, 161.6. Anal. ($C_{19}H_{23}ClN_8$) C, H, N.

**7-Benzylamino-6-chloro-2-(2-hydroxyethylamino)-4-pyrrolidinopteridine (7l).** See **7c**. Preparation started with 195 mg (0.52 mmol) of **6a** in 10 mL of dioxane. Ethanolamine (260 $\mu$L, 4.3 mmol) was added, and the mixture was refluxed for 5 h. Yield was 181 mg (87.1%), and yield after column chromatography (silica gel, ethyl acetate/ethanol = 5/1 v/v) was 117 mg (56.4%); pale-yellow crystals, mp 198 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.92 (br, 2), 2.01 (br, 2), 3.61−3.65 (m, 2), 3.72 (br, 2), 3.81 (t, $^3J$ = 4.6 Hz, 2), 4.11 (br, 2), 4.77 (d, $^3J$ = 4.9 Hz, 2), 5.75 (t, $^3J$ = 4.9 Hz, 1), 5.93 (br, 1), 7.29−7.37 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.8, 26.8, 44.7, 45.4, 49.6, 50.7, 64.5, 115.2, 126.1, 127.7, 128.0, 128.8, 137.7, 151.4, 157.0, 157.2, 162.0; MS *m/e* 399 ($M^+$). Anal. ($C_{19}H_{22}ClN_7O$) C, H, N.

**7-Benzylamino-6-chloro-2-(dimethylamino)-4-pyrrolidinopteridine (7m) and 7-Benzylamino-2-chloro-6-(dimethylamino)-4-pyrrolidinopteridine (7n).** In a 60 mL

glass tube for a maximum pressure of 20 bar, 386 mg (1.03 mmol) of **6a** were suspended in 25 mL of DMF. Gaseous ammonia was passed for 1 min through the tube, then the system was closed, and the tube was heated under thorough stirring with a magnetic stirrer at 100 °C for 4 h. Thereafter the pressure was removed, and the mixture was poured into 100 mL of water. The precipitate was collected on a filter, washed with 2% $NH_3$ and water, and dried. Column chromatography on silica gel (∅ 2.5 cm; $l$ = 70 cm; ethyl acetate/hexane = 1/1 v/v) yielded 112 mg (28.4%) of **7n** with $R_f = 0.55$ and 151 mg (38.2%) of **7m** with $R_f = 0.30$.

**7-Benzylamino-6-chloro-2-(dimethylamino)-4-pyrrolidinopteridine (7m):** light-yellow needles, mp 196 °C; $^1$H NMR ($CDCl_3$) $\delta$ 1.93 (br, 2), 2.01 (br, 2), 3.24 (s, 6), 3.76 (br, 2), 4.11 (br, 2), 4.83 (d, $^3J$ = 4.9 Hz, 2), 5.62 (t, $^3J$ = 4.9 Hz, 1), 7.30−7.37 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.8, 27.2, 37.3, 45.6, 49.2, 50.2, 114.8, 125.3, 127.7, 128.1, 128.8, 138.0, 151.3, 156.9, 157.5, 161.2; MS *m/e* 383 ($M^+$). Anal. ($C_{19}H_{22}ClN_7$) C, H, N.

**7-Benzylamino-2-chloro-6-(dimethylamino)-4-pyrrolidinopteridine (7n):** fine colorless needles, mp 187 °C; $^1$H NMR ($CDCl_3$) $\delta$ 1.93 (br, 2), 2.02 (br, 2), 2.79 (s, 6), 3.77 (br, 2), 4.26 (br, 2), 4.79 (d, $^3J$ = 5.5 Hz, 2), 5.77 (t, $^3J$ = 5.5 Hz, 1), 7.29−7.38 (m, 5); $^{13}$C NMR ($CDCl_3$) $\delta$ 23.7, 27.0, 41.1, 45.3, 49.6, 50.7, 115.8, 127.6, 128.1, 128.8, 138.0, 145.9, 149.8, 154.7, 157.4, 157.9; MS *m/e* 383 ($M^+$). Anal. ($C_{19}H_{22}ClN_7$) C, H, N.

**7-Benzylamino-2-piperazino-4-pyrrolidinopteridine (7o).** A suspension of 356 mg (0.84 mmol) of **7a** in 7.5 mL of ethanol was stirred vigorously with 2.5 mL of 0.5 N HCl containing 350 mg of 10% Pd/C for 2 h with a slight excess pressure of $H_2$. Then water (15 mL) and ethanol (25 mL) were added, the mixture was filtered, and the ethanol was removed in vacuo. The aqueous solution was adjusted to pH 9 with 0.2 N $NH_4OH$, and the precipitate was allowed to stand overnight. The solid was filtered, washed with water, and dried in vacuo to afford 259 mg (79.1%) of crude product. Column chromatography (silica gel, ethanol + 2.5% triethylamine) yielded 178 mg (54.4%) of light-yellow crystals of **7o**; mp 218 °C: $^1$H NMR ($CDCl_3$) $\delta$ 1.77 (s, 1), 1.93 (br, 4), 2.79 (t, $^3J$ = 5.0 Hz, 4), 3.88 (t, $^3J$ = 5.0 Hz, 4), 4.70 (d, $^3J$ = 5.6 Hz, 2), 5.29 (br, 1), 7.24−7.33 (m, 5), 7.58 (s, 1); $^{13}$C NMR ($CDCl_3$) $\delta$ 24.0, 45.1, 45.3, 46.2, 49.8, 117.0, 126.6, 127.5, 127.8, 128.7, 138.3, 155.2, 157.7, 157.9, 160.3; MS *m/e* 390 ($M^+$). Anal. ($C_{21}H_{26}N_8$) C, H, N.

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