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Review Article

The synthesis and biological evaluation of new methotrexate derivatives in rheumatoid arthritis

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CONTENTS

Introduction 1015
Methotrexate toxicity and polyglutamation 1015
Methotrexate derivatives in rheumatoid arthritis 1016
Nonpolyglutamatable derivatives 1016
Polyglutamatable derivatives 1017
Synthesis of antiarthritic derivatives 1018
Method A 1018
Method B 1018
Future directions 1020
References 1020

Introduction

RA is known to be a chronic inflammatory disease of joints with autoimmune features, characterized by synovial proliferation and articular destruction. Drug therapy for rheumatoid arthritis (RA) has consisted of nonsteroidal antiinflammatory drugs (NSAIDs), corticosteroids and disease modifying antirheumatic drugs (DMARDs). NSAIDs such as aspirin and indomethacin are cyclooxygenase inhibitors and are used as first-line drugs. DMARDs such as gold compounds and penicillamin are used as second-line drugs; however, several or more weeks elapse after initiation of these drugs before clinical improvement occurs. These are called slow-acting antirheumatic drugs. However, NSAIDs and DMARDs show little or no effect on the course of disease progression and have severe side effects. Corticosteroids are currently the most effective at ameliorating disease progression, but they show severe side effects during long-term use. Therefore, drugs which are safe and have greater potency have been sought. In the mid-1980s, methotrexate (MTX; **1**, Fig. 1) at low weekly dosages was established as a more effective and relatively safe therapy for RA patients who had failed to respond to conventional therapy with anti-RA drugs (1). The antirheumatic effects of MTX were apparently faster (within 4 weeks after initiation of therapy) compared to other drugs and its efficacy reaches maximum between 6 and 12 months after starting treatment (2, 3). Furthermore, in recent years, MTX has been used increasingly to treat early stages of the disease as a first-line drug. Consequently, its efficacy and safety for long-term treatment are now better defined.

Methotrexate toxicity and polyglutamation

In spite of the excellent efficacy of MTX, gastrointestinal side effects, hepatotoxicity and pulmonary toxicity are major problems during clinical use. Gastrointestinal side effects such as anorexia, nausea, vomiting and diarrhea are very common and their frequency may be as high as 60% (4). Although they are not serious, they are the major reason for discontinuation of therapy (5). Transient and mild increases in liver enzymes such as GOT and GPT are observed in about 20% of patients receiving MTX. On rare occasions severe cirrhosis can occur (6, 7), and hepatic fibrosis occurs with long-term MTX therapy (about 50% for 53 months) (8). Interstitial pneumonitis is one of the most unpredictable and life-threatening side effects of MTX therapy (9), although fortunately its incidence is < 5%. Other side effects are stomatitis, central nervous system toxicity (*e.g.*, headache) and hematological toxicity (*e.g.*, leukopenia).

MTX undergoes polyglutamation intracellularly by the enzyme folypolyglutamate synthetase (FPGS) (10). The enzyme adds residues of glutamic acid in gamma peptide linkage to the glutamyl moiety of MTX. Polyglutamation increases molecular weight, polarity and affinity to folate-dependent enzymes such as thymidylate synthase, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICARtf) and dihydrofolate reductase (DHFR); consequently, MTX's pharmacological effects are potentiated (11, 12). However, this causes the accumulation of MTX polyglutamates in large amounts within cells which are retained for longer periods than MTX itself, resulting in cell death via folate depletion (13). These characteristics of MTX have suggested that polyglutamation plays an essential role in the development of side effects.

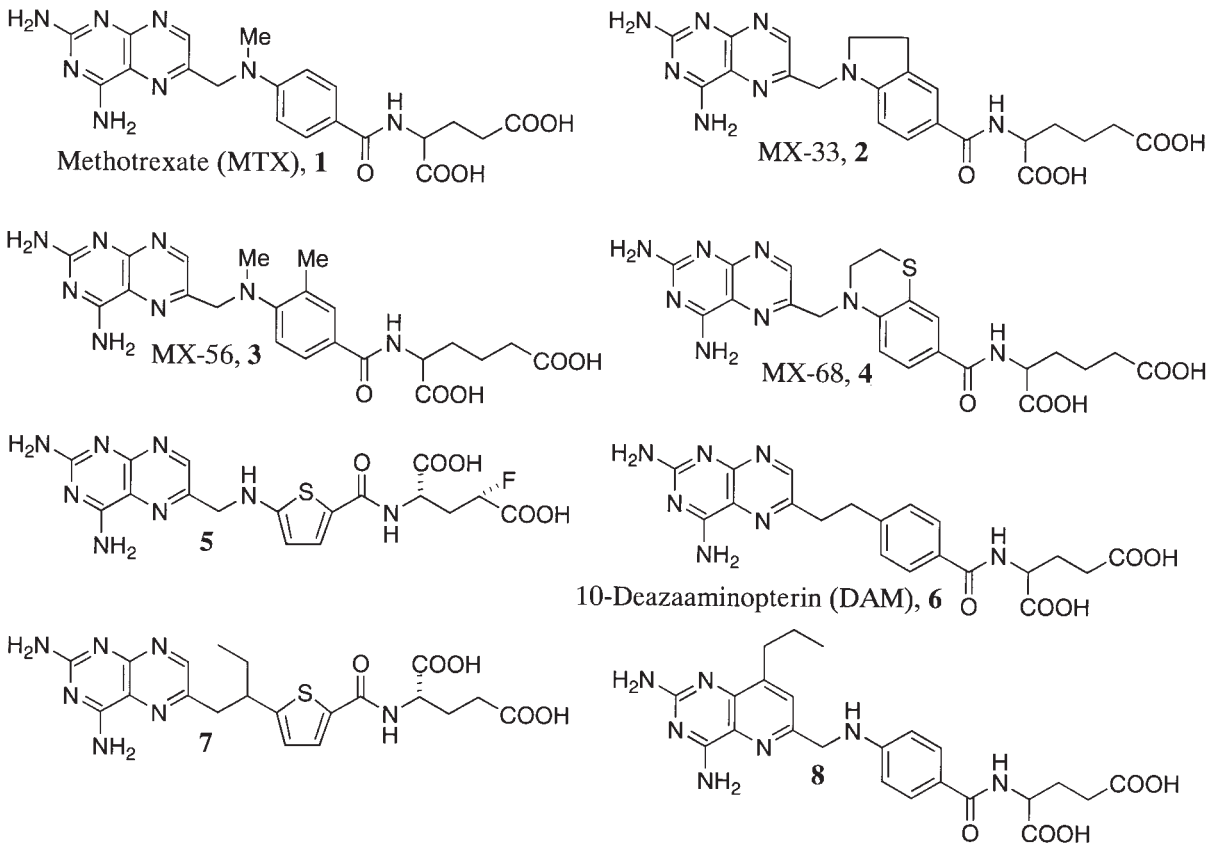


Fig. 1. Methotrexate and its antiarthritic derivatives.

Methotrexate derivatives in rheumatoid arthritis

In 1995, DeGraw published an excellent review of this area (14). After his publication, a considerable number of reports of medicinal chemistry in this area were published. In this section, we will review newly synthesized MTX derivatives developed as antirheumatoid agents during the last 3 years; these have been categorized into two classes of compounds based on their biochemical transformations, namely, nonpolyglutamatable and polyglutamatable MTX derivatives.

Nonpolyglutamatable derivatives

MTX derivatives which do not undergo polyglutamation were originally developed for cancer chemotherapy (15-17). Rosowsky has already reported various syntheses and biological evaluations of the nonpolyglutamatable MTX derivatives bearing various amino acid moieties (18-30).

As mentioned above, polyglutamation is thought to be responsible not only for the pharmacological potentiation of MTX but also for its side effects during therapy. With this in mind, medicinal chemists in the field of rheumatology have focused their efforts on the synthesis of nonpolyglutamatable MTX derivatives.

Our group initiated work on a nonpolyglutamatable form of MTX in order to produce a safer antirheumatic agent. *In vitro* biological evaluations of synthesized compounds were performed using antiproliferative assays on human synovial cells (hSC) and peripheral blood mononuclear cells (hPBMC) obtained from patients with RA and healthy volunteers, respectively. Some compounds were further evaluated as an antirheumatic agent using adjuvant-induced (AIA) and collagen type II-induced arthritis (CIA) models. First of all, we modified the aminobenzoic acid moiety of MTX and found that the resulting indoline analog had more potent effects than MTX both in *in vitro* and *in vivo* assays (31). Subsequently, the amino acid moiety of MTX was replaced with some other amino acid having no COOH group at the γ-position (32). As a result of these modifications, MX-33 (**2**, Fig. 1), having a homoglutamate, was found to have potent antiproliferative and considerable antiarthritic effects when administered orally. In addition, MX-33 was shown to undergo polyglutamation *in vitro* using Moran's method (33). A structurally related compound, MX-56 (**3**, Fig. 1), exhibited less potent antiarthritic effects than MX-33, but did show similar *in vitro* effects

(34). One of the possible causes of the depressed *in vivo* effect was thought to be the consequence of its enhanced hydrophobicity, resulting in inefficient penetration in the small intestine.

In order to further enhance the pharmacological efficacy of MX-33, close attention was paid to the indoline moiety. After considering Oefner's model (35) and our own binding studies (36), we predicted that we could potentiate binding to DHFR without changing the direction and orientation of the pteridine ring if we replaced the indoline ring of MX-33 with a slightly enlarged ring. In our model, the carbon atom adjacent to the phenyl ring in this indoline moiety was partially exposed to the solvent portion; therefore, insertion of either an oxygen or a sulfur atom between the methylene group and benzene ring was expected to energetically favor its location toward the solvent portion and gain a more stable interaction with solvent water molecules rather than the carbon atom. In particular, insertion of a sulfur atom was expected to enhance DHFR binding by van der Waals interactions. Thus, MX-68 (**4**, Fig. 1), having a dihydrobenzothiazine ring and homoglutamate, was designed and prepared (37).

As expected, MX-68 potently suppressed the proliferation of several kinds of cells. Its antiproliferative effect against hPBMC, hSC and human endothelial cells (hEC) was almost identical to those of MTX (38). This suppressive effect of MX-68 was completely reversed by the coaddition of folinic acid, which is commonly used in rescue therapy after high doses of MTX, suggesting that MX-68 has similar characteristics to MTX. The major biochemical difference between MX-68 and MTX is that MX-68 does not undergo polyglutamation, whereas MTX does. Taking this into consideration, MX-68 is easily expected to be released from the cells after its removal from the environment. In fact, when MX-68 was removed during cell culture, the antiproliferative effect completely disappeared, whereas that of MTX still existed, although it was weakened (38). MX-68 also showed potent antiarthritic effects in several arthritis models in rodents. In mouse CIA, MX-68 orally and dose-dependently suppressed the onset of arthritis. Furthermore, administration of 2 mg/kg of MX-68 completely suppressed arthritis, when it was given orally 3 times a week from the day of first collagen immunization. In contrast, MTX did not show significant suppression at a dose of 2 mg/kg (38).

MX-68 also significantly suppressed the onset of autoimmune disease and prolonged survival in lupus mice such as MRL/lpr mice and NZB/W F1 mice, when it was administered orally 3 times a week, starting from a young age before the onset of disease (39, 40). We evaluated the immunosuppressive activity of MX-68 compared with MTX *in vivo*. MX-68 suppressed antigen-specific antibody production and delayed-type hypersensitivity reaction dose-dependently. These suppressive effects were slightly stronger than those of MTX (41).

The MX-68 derivatives, MX-33 and MX-56, showed similar suppressive effects on hPBMC proliferation, although they were varied against hSC and hEC. Briefly, MX-33 was somewhat weaker and MX-56 was slightly stronger than MTX. In rat AIA, MX-33 and MX-56 were less effective than MX-68 (34, 37).

Radiolabeled MX-68 has already been prepared (42), and MX-68 is currently under preclinical investigation with the aim of beginning a clinical study in the near future.

Kokuryou *et al.* reported the biological profiles of newly synthesized MTX derivatives bearing γ-fluoroglutamate (43). Importantly, the fluorinated MTX (FMTX) derivatives are expected not to be polyglutamated. The authors speculated that the suppressed polyglutamate formation was probably due to the extreme electronegativity of fluorine causing acidity enhancement of the γ-COOH group. The acidity of γ-COOH group in structurally different MTX derivatives was also suggested to play an important role in polyglutamation.

One of the FMTX derivatives, **5** (Fig. 1), inhibited the proliferative responses of mouse spleen cells to concanavalin A or lipopolysaccharide. These effects were 2-3 times stronger than those of MTX. However, the suppressive effect of **5** on primary antibody production *in vivo* was much weaker than that of MTX, when administered orally. Compound **5** also suppressed the development of rat AIA. ED_{50} values of **5** and MTX were 0.8 and 0.058 mg/kg, respectively, when they were given orally 5 times a week beginning the day after adjuvant injection (total 12 days). However, the safety index (LD_{50}/ED_{50} value) of **5** was 4 times higher than that of MTX.

Recently, Hart *et al.* and Tsukamoto *et al.* reported the synthesis and biological evaluation of β,β-difluoro MTX derivatives and γ,γ-difluoro folic acid derivatives, respectively (44, 45). However, their antiarthritic effects were not apparent.

Polyglutamatable derivatives

Galivan *et al.* reported that MTX and aminopterin inhibited inflammation in rat arthritis models, whereas other derivatives lacking a pteridine ring or a glutamate residue did not (46). Alarcon *et al.* investigated the antiarthritic efficacy of 10-deazaaminopterin (DAM; **6**, Fig. 1) in patients with RA (47). This study confirmed its similar efficacy and toxicity to that of MTX, which was consistent with a comparative study using a MRL/lpr mouse, a model of spontaneous autoimmune disease (48).

As part of an extensive program for synthesis and biological evaluation of new MTX derivatives in RA, DeGraw *et al.* (49) and Piper *et al.* (50) reported the synthesis, biological evaluation and resulting structure-activity relationship (SAR) of 10-deazaaminopterin and 5-deazaaminopterin derivatives, respectively. The 10-deazaaminopterin derivatives, replaced with some heteroarylcarbonyl moiety in place of the aminobenzoic acid moiety in MTX, were synthesized and tested for *in vitro* antiproliferative effects using a mouse leukemia cell line (L1210), and were evaluated for antiarthritic efficacy in a mouse CIA model. Among them, thienyl derivatives **7** (Fig. 1) were found to have the most potent antiproliferative

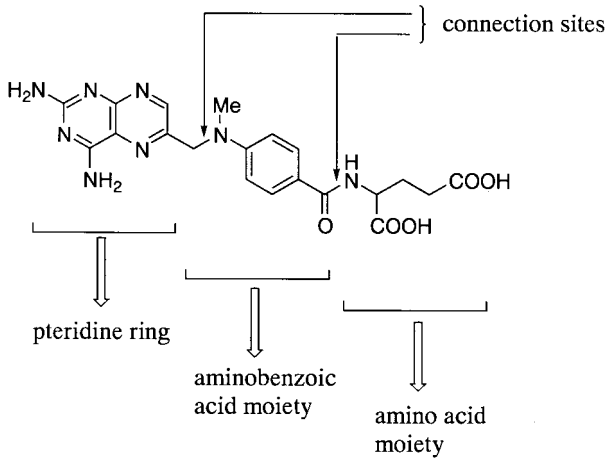


Fig. 2. Structural division of methotrexate.

(IC_{50} = 3.7 nM) and antiarthritic activity. The 5-deaza-aminopterin derivatives were also synthesized and tested using the same method as described for the biological evaluation of the 10-deaza derivatives. In particular, 5-deaza-5-*n*-propylaminopterin **8** (Fig. 1) was suggested to be the most promising candidate. It has a potent antiproliferative effect (IC_{50} = 3.7 nM) and comparable antiarthritic efficacy to MTX, with a 12-13 times higher LD_{50} value than MTX.

Synthesis of antiarthritic derivatives

From a synthetic point of view, the structure of MTX is divided into three parts, *i.e.*, pteridine, aminobenzoic acid and amino acid moiety (Fig. 2). Synthesized anticancer and antiarthritic MTX derivatives have basically similar structural components as MTX. Thus far, two alternative synthetic routes have been reported using a synthetic intermediate corresponding to the three substructures. One is an initial alkylation of the aminobenzoic acid moiety with the pteridine moiety, followed by an amide formation with an amino group of the amino acid moiety (Method A). The other occurs by inverse order of these steps, that is, an initial amide formation of an N-protected aminobenzoic acid moiety with an amino acid moiety, followed by the deprotection and alkylation of the deprotected amino group with the pteridine moiety (Method B).

Here, we would like to describe one example for each of the synthetic routes.

Method A

Compounds **6-8** (Fig. 1) and other classical antifolates were prepared according to Method A (51-55), one of which is the synthetic route of **8** (50).

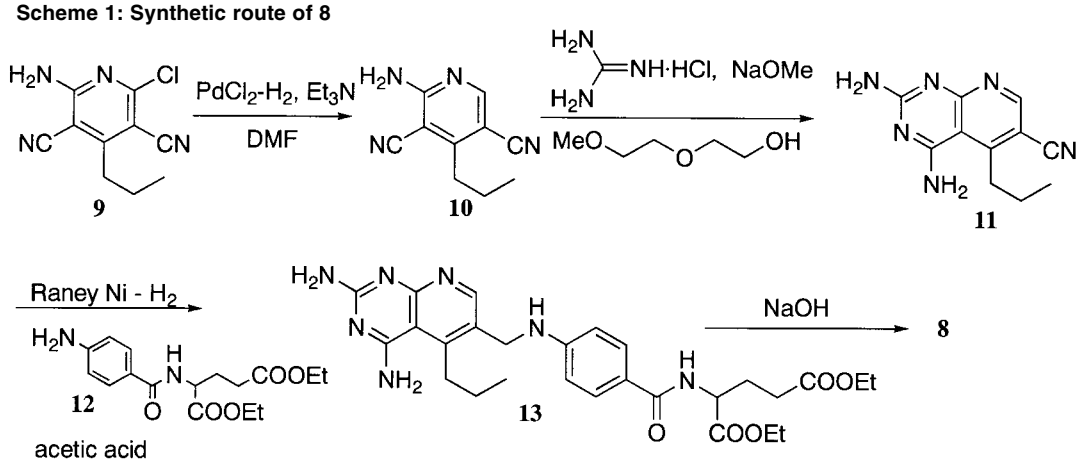
As shown in Scheme 1, 2-amino-4-propyl-6-chloropyridine-3,5-dicarbonitrile (**9**) is initially subjected to palladium catalytic hydrogenation to give a dechlorinated pyridine **10** (56). Subsequently, a condensation of **10** with guanidine gives 5-deazapteridine (**11**). A reductive amination of **11** with **12** is performed, using Raney Ni in acetic acid under hydrogen atmosphere, to give **13**. Finally, hydrolysis of **13** produces the target compound **8**.

Method B

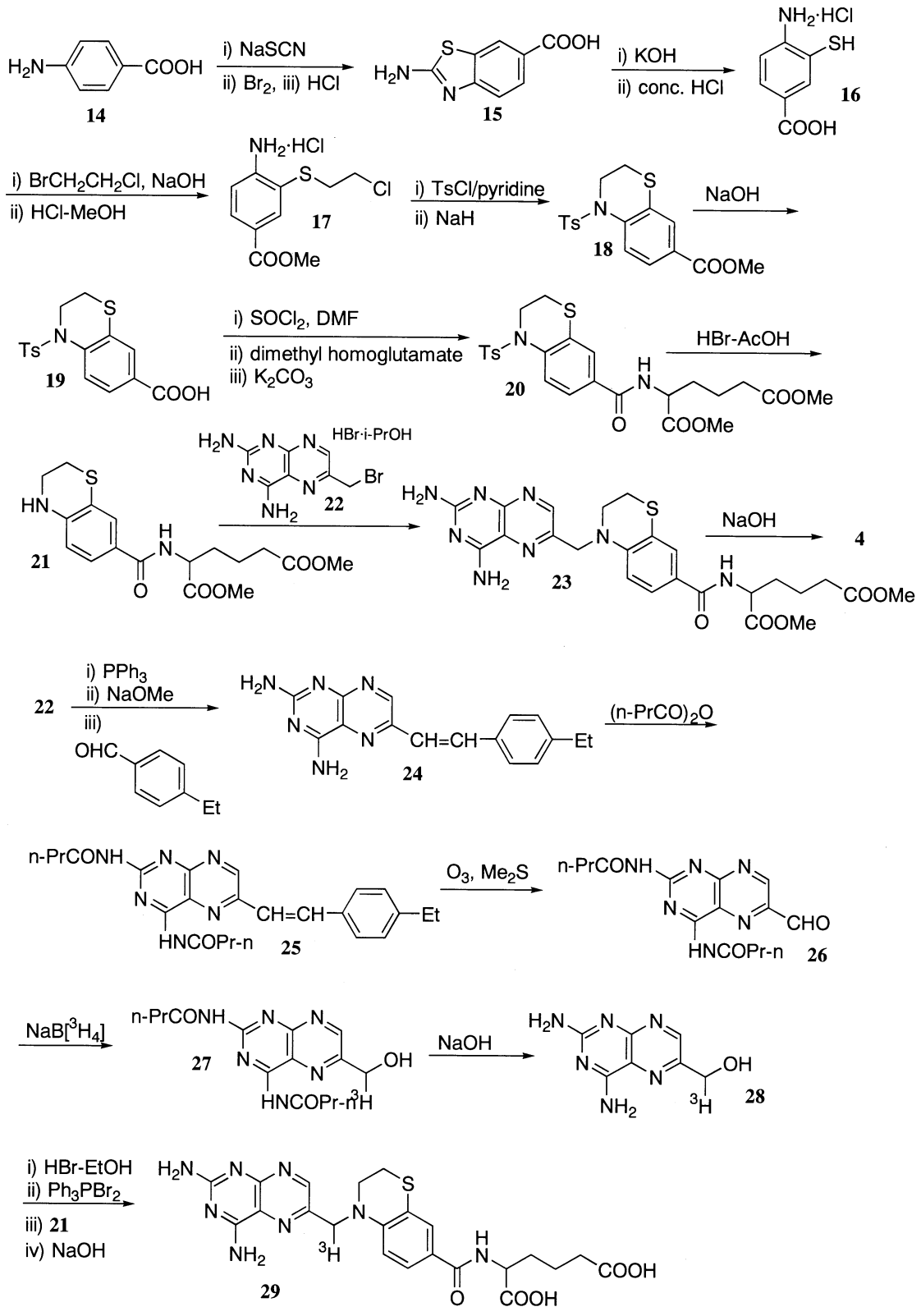
Compounds **2-5** (Fig. 1) and other classical antifolates were prepared using Method B (57-60), one of which is the synthesis of **4** (MX-68) and its tritiated derivative **29** (37, 42).

As shown in Scheme 2, initially, starting material **14** is converted to benzothiazol **15** by treatment of sodium thiocyanate in the presence of bromine. Alkali hydrolysis of **15** gives thiol **16**, which is immediately submitted to S-alkylation with 1-bromo-2-chloroethane, followed by acid esterification, which gives thioether **17**. After protection of the amino group of **17** with tosyl chloride, treatment

Scheme 1: Synthetic route of 8



Scheme 2: Synthetic routes of 4 (MX-68) and its tritiated derivative



of NaH affords benzothiazine ester **18**, and next hydrolysis produces the key intermediate **19**. Subsequently, **19** is converted to the corresponding acid chloride and amidated with dimethyl homoglutamate to give the amide **20**. The tosyl group of the amide **20** is removed with HBr-AcOH to give the amine **21**, which is effectively alkylated with 6-(bromomethyl)-2,4-diaminopteridine (**22**) to produce **23**. Finally, **23** is hydrolyzed with 1N NaOH to yield **4** (MX-68).

The synthetic scheme of tritiated MX-68 (**29**) is also presented in Scheme 2. Treatment of **22** with triphenylphosphine, followed by Wittig reaction with 4-ethylbenzoic aldehyde yields an olefin **24**. To improve the solubility of **24** in routine organic solvents, the amino group of **24** is acylated to give a lipophilic olefin **25**. The olefin moiety is next oxidized by treatment of ozone to give an aldehyde **26**. Reduction of **26** with $NaB[^3H_4]$ is performed in isopropanol to give the corresponding tritiated alcohol **27**. The alcohol **27** is deacylated to yield acyl-free pteridine **28**. The conversion of **28** to HBr salt, followed by bromination of alcohol with dibromotriphenylphosphorane, amination with **21** and alkali hydrolysis produces $[9\text{-}^3H_1]$MX-68 (29).

Future directions

Since MTX was synthesized 50 years ago, medicinal chemists in the area of cancer chemotherapy have focused their efforts on enhancing the pharmacological effects of MTX, including anti-DHFR activity, antiproliferative effects on cancer cells and avoidance of drug resistance. The notable antiarthritic efficacy of pulse administration of low-dose MTX has created more interest in MTX derivatization. MTX derivatives are promising candidates as antiarthritic agents because of their potent antiproliferative effects in synovial cells and lymphocytes, but not in cancer cells. In addition, they cause fewer adverse events, such as lung fibrosis and hepatic dysfunction, normally associated with long-term therapy of RA. Some of the newer MTX derivatives discussed here will enter clinical trials in the near future and should be beneficial in treating RA.

Recently, Johnson *et al.* (61) and Cody *et al.* (62) analyzed the NMR solution structure and the crystal structure of human DHFR ternary complex of NADPH with PT523, a potent antitumor MTX analog, respectively. These successful analyses also provide new directions for MTX derivatization from a structural point of view.

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