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# Synthesis and Evaluation of Hydrogen Peroxide Sensitive Prodrugs of Methotrexate and Aminopterin for the Treatment of Rheumatoid Arthritis

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ABSTRACT. A series of novel hydrogen peroxide sensitive prodrugs of methotrexate (MTX) and aminopterin (AMT) were synthesized and evaluated for therapeutic efficacy in mice with collagen induced arthritis (CIA) as a model of chronic rheumatoid arthritis (RA). The prodrug strategy selected is based on ROS-labile 4-methylphenylboronic acid promoieties linked to the drugs *via* a carbamate linkage or a direct C–N bond. Activation under patho-physiological concentrations of $H_2O_2$ proved to be effective and prodrug candidates were selected in agreement with relevant *in vitro* physicochemical and pharmacokinetic assays. Selected candidates showed moderate to good solubility, high chemical and enzymatic stability, and therapeutic efficacy comparable to the parent drugs in the CIA model. Importantly, the prodrugs displayed the expected safer toxicity profile and increased therapeutic window compared to MTX and AMT while maintaining a comparable therapeutic efficacy, which is highly encouraging for future use in RA patients.

KEYWORDS: aminopterin, collagen-induced arthritis, hydrogen peroxide, methotrexate, phenylboronic acid, prodrug, rheumatoid arthritis.

## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic destructive synovitis and is associated with progressive disability, systemic difficulties, premature death, and socioeconomic costs.[1] The cause of RA remains unknown and currently no cure for the disease exists. Several studies have estimated the prevalence of RA to range between 0.5% to 1% of the adult population in developed countries, with a 3 to 1 female to male ratio.[2]

Aminopterin (**1**, AMT, Figure 1), an anti-folate drug initially developed for treatment of leukemia, was tested for RA treatment in 1951.[3,4] Complex manufacturing,[5] unpredictable toxicities,[6–9] and low therapeutic index[8,10] led scientist to develop methotrexate (**2**, MTX, Figure 1), as an alternative antifolate for cancer therapy which later showed anti-arthritic properties as a disease modifying anti-rheumatic drug (DMARD). In 1988, low-dose MTX (LD-MTX, <30 mg in weekly pulses) was approved by the FDA for the treatment of RA and has remained the standard of care since.[11,12] Although efficacious, the mechanisms that govern the anti-inflammatory and immunosuppressive effects of MTX in rheumatic diseases are still not completely understood.[13] Furthermore, the population of patients that will be responsive to an MTX regiment cannot be reliably predicted before initiating treatment.[14] Adverse effects and low efficacy of LD-MTX in RA are the predominant reasons for discontinuation of treatment. Side effects are usually mild, self-limiting, and/or preventable, but a subset of patients suffers from severe side-effects. Toxicities including anemia, neutropenia, stomatitis, oral ulcers, lethargy, fatigue, nodulosis, hepatic and pulmonary fibrosis, as well as renal insufficiency are associated to this drug. [15–18] Additionally, progressive drug tolerance of disease, high patient variability, and pharmacokinetic inadequacies of LD-MTX suggest the need of more efficient and safer RA therapies.

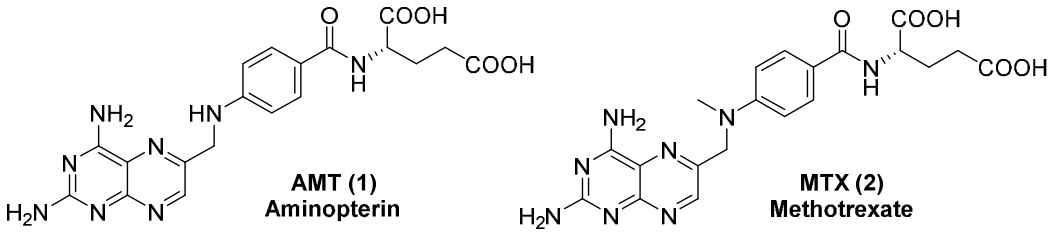



**Figure 1.** Chemical structure of aminopterin (AMT, **1**) and methotrexate (MTX, **2**)

Prodrugs are chemically modified drugs that have either no or greatly decreased activity compared to the parent drug. Upon administration, prodrugs are activated in the body through chemical and/or enzymatic transformation into the pharmaceutically active moiety.[19] Approximately 20% of drugs approved since 2000 are prodrugs,[20] mostly designed to minimize and/or overcome unacceptable absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of the native drug.[21] Several prodrugs, drug conjugates, and drug delivery systems for methotrexate have been reported in the literature but none of them have been approved for clinical use yet. [22–33]

Reactive oxygen species (ROS), like $H_2O_2$, $HO^{\bullet}$, $O_2^{-}$, produced by phagocytes and neutrophils,[34] have important physiological roles in priming the immune system.[35,36] Increasing evidence from different studies supports the relation between oxidative stress and the pathogenesis of inflammation and rheumatoid arthritis.[37,38] In RA, oxidative stress has been described as an important mechanism in the pathogenesis of destructive proliferative synovitis. Under pathological inflammatory conditions, the extracellular concentration of $H_2O_2$, the most stable ROS, has been determined to be up to a 100-fold higher compared to healthy tissue, reaching concentrations as high as 1.0 mM.[39–44] The presence of increased concentrations of $H_2O_2$ in the inflammatory environment can serve as the stimulus for prodrug activation in site-

selective drug delivery systems. Phenylboronic acids and esters,[45–48] phenylsulfonate esters,[49] thiazolidinones,[44] *N*-(2,5-dihydroxyphenyl)acetamides,[50] and α-boryl ethers, carbonates, and acetals[51] have been reported as useful structural motifs for hydrogen peroxide activatable prodrugs and imaging, but studies have mainly focused on their applications in oncology.

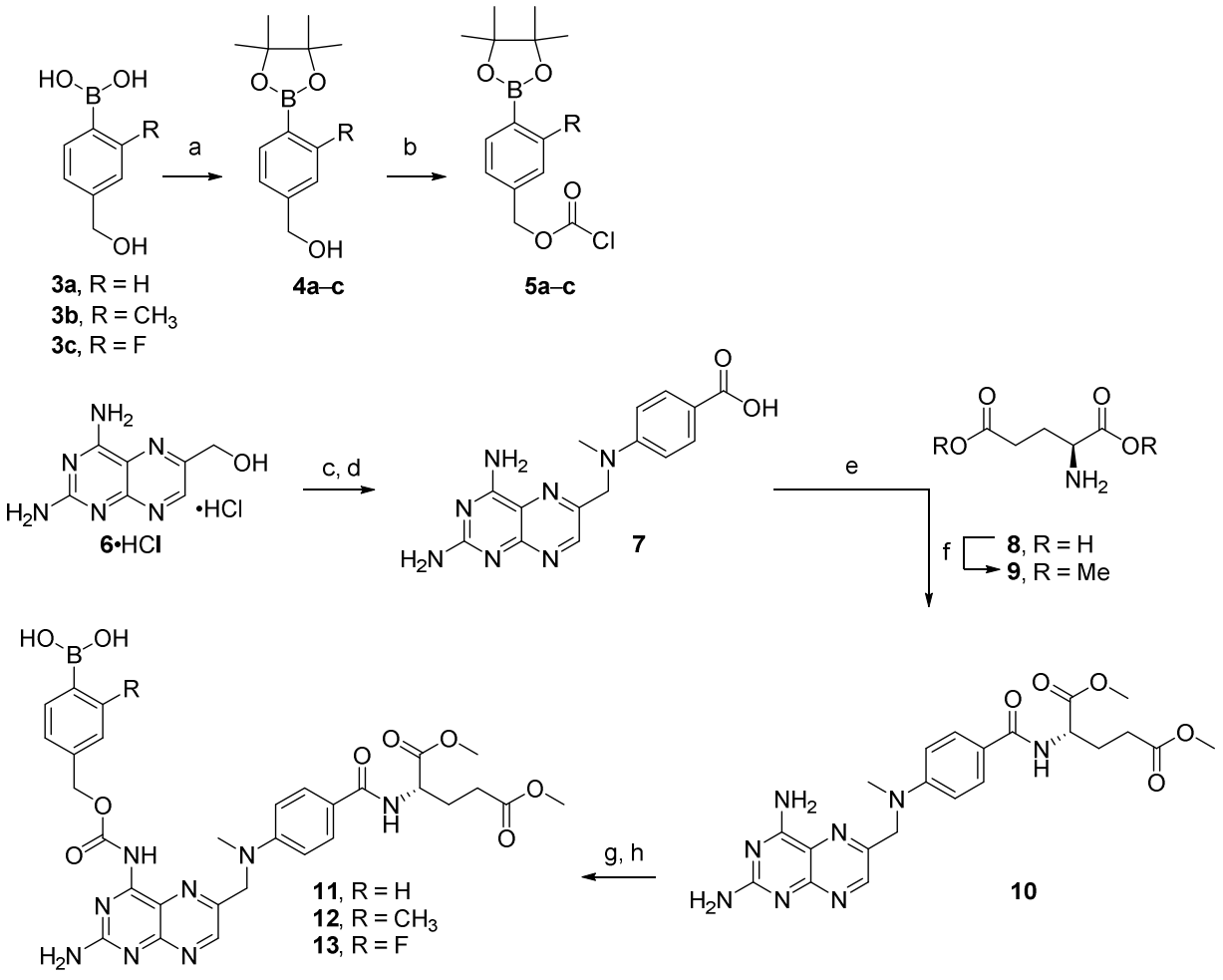
Here we propose a new approach to RA therapy based on the use of phenylboronic acid-containing prodrugs that are inactive until activated locally in the inflammatory tissue. This mechanism secures that the biological activity is limited to regions of inflammation to facilitate a safer toxicity profile compared to AMT and MTX, and a more effective treatment due to the expected localization and accumulation in the target tissue. We have designed and synthesized a series of MTX and AMT prodrugs that are activated by disease- and tissue-specific factors like $H_2O_2$. Their physicochemical, pharmacokinetic, and drug-like properties have been evaluated in *in vitro* assays. Additionally, their *in vivo* efficacy and preliminary toxicity in the murine collagen-induced arthritis model (CIA) was assessed.

## RESULTS AND DISCUSSION

**Synthesis of prodrugs.** The pinacolates **4b** and **4c** were synthesized from the corresponding 4-hydroxymethylphenyl boronic acids **3b** and **3c** *via* condensation with pinacol (Scheme 1). Subsequently, the chloroformates **5a–c** were achieved by reaction of **3a–c** with phosgene. In parallel, the MTX core structure was synthesized starting with bromination of 2,4-diamino-6-(hydroxymethyl)pteridine hydrochloride (**6**·HCl) and alkylation of 4-methylaminobenzoic acid to form the pteoric acid **7**, following the procedure described by Kralovej *et al*.[52] Amide coupling of **7** with dimethylglutamate hydrochloride **9**·HCl, synthesized from glutamic acid **8**, provided methotrexate dimethylester **10**. Finally, coupling between **10** and **5a-c** formed the desired

prodrugs **11–13** after hydrolysis of the pinacolate with HCl in a mixture of $H_2O$/MeCN. The use of DMAP in the coupling step was crucial for the progression of the reaction. Compounds **11–13** represent a set of MTX prodrugs in which the promoiety is linked to MTX via a carbamate linkage. The promoiety contains different *ortho*-substituents (H, F or Me) in order to investigate the effect of modifying the steric and electronic environment around the boronic acid on reactivity towards $H_2O_2$ . Furthermore, the carboxylic acids of MTX were also masked as methyl esters.

**Scheme 1.** Synthesis of MTX prodrugs **11–13**[a]



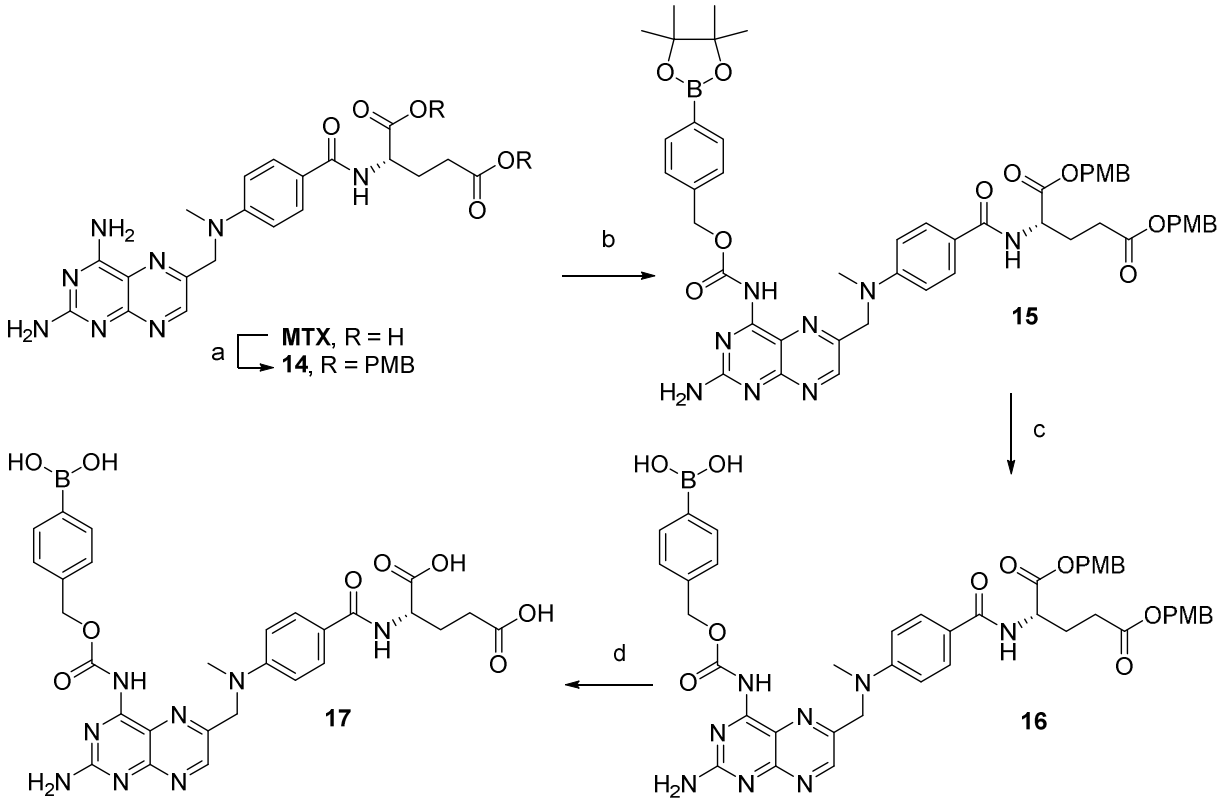

[a]Reagents and conditions: (a) pinacol, THF, reflux, 16 h (>94%). (b) $COCl_2$ (20% in toluene), dioxane, 21 $^oC$, 20 h (>95%). (c) NaOH, $H_2O$. (d) $PPh_3Br_2$, DMA, 21 $^oC$, 20 h, then

4-methylaminobenzoic acid, DIPEA, 3 days (77%). (e) PyBOP, $Et_3N$, DMF, 21 °C, 30 min, then **9**·HCl (86%). (f) MeOH, $SOCl_2$, 21 °C, 4 days (>95%). (g) **5a–c**, DIPEA, DMAP, $CH_2Cl_2$, 21 °C, 5 h. (h) HCl in $H_2O$/MeCN, 21 °C, 16 h (27–32%).

Efforts aiming for hydrolysis of the methyl esters in **11–13** using standard conditions based on alkaline hydrolysis with NaOH[53] or LiOH,[54] LiI in pyridine,[55] or enzymatic hydrolysis using a polymer supported lipase from *Candida antarctica*[56] were unsuccessful. Therefore a new synthetic strategy was proposed (see Scheme 2).

Starting from MTX, the synthesis of the corresponding PMB protected intermediate **14** was achieved. Following the same procedure described for the synthesis of **11–13**, coupling between **5a** and **14** afforded **15**, which was hydrolyzed to form the corresponding boronic acid **16**. Lastly, PMB deprotection under acidic conditions afforded the prodrug **17**.

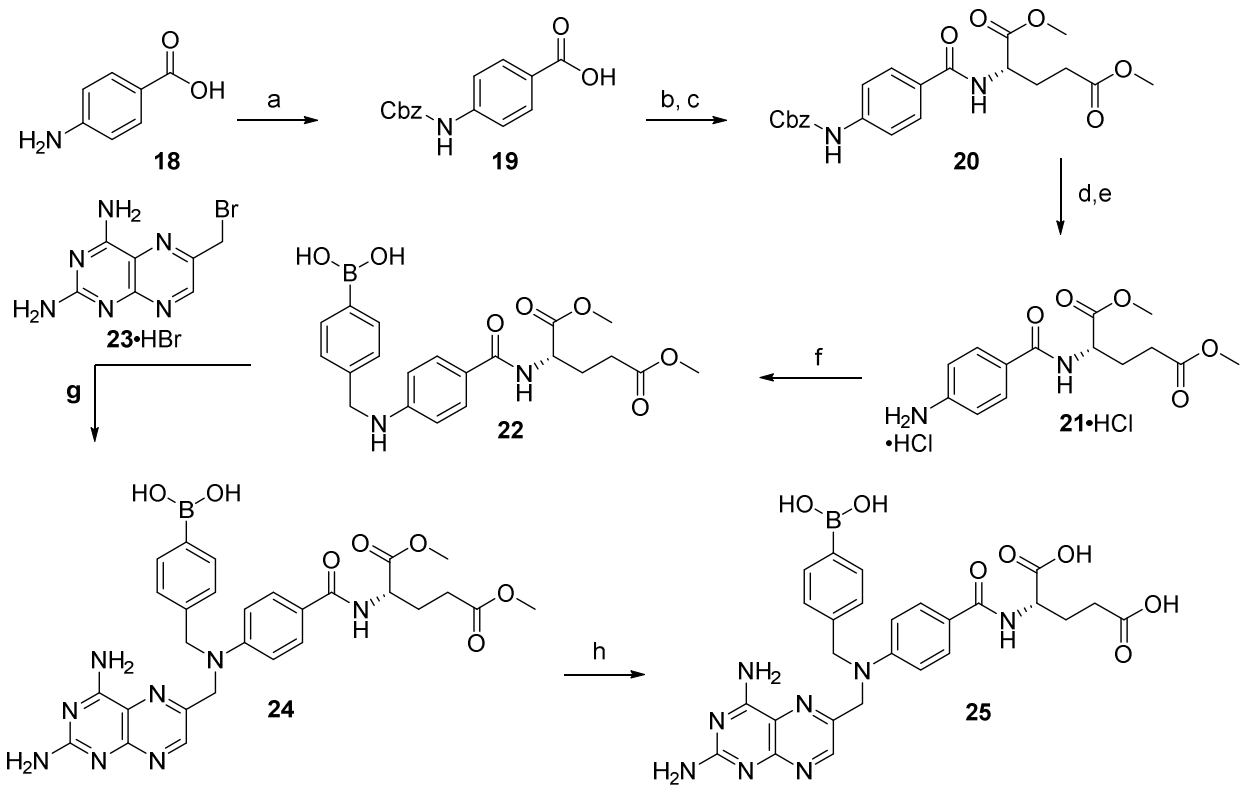
**Scheme 2.** Synthesis of MTX prodrug **17**[a]



[a]Reagents and conditions: (a) PMBCl, 1,1,3,3-tetramethylguanidine, $CH_2Cl_2$, 21 °C, 16 h (35%). (b) **5a**, DIPEA, DMAP, $CH_2Cl_2$, 21 °C, 5 h (73%). (c) HCl in $H_2O$/MeCN, 21 °C, 20 h (31%). (d) 5% TFA in $CH_2Cl_2$, 21 °C, 30 min (54%).

Cbz protection of 4-aminobenzoic acid (**18**) formed **19** and two-step coupling with **9**·HCl produced **20**. Hydrogenolysis of **20** afforded **21** in high yields, which was subjected to reductive alkylation with 4-formylphenylboronic acid to give **22**. Alkylation of **22** with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**23**·HBr) formed the first AMT prodrug **24**. The concentration of the reaction was critical to obtain a reasonable yield, as previously published by Montgomery *et al.*[57] for similar substrates. The last step consisted of alkaline hydrolysis of **24** to form the second AMT prodrug **25**. These compounds represent the proposed set of phenylboronic acid-based AMT prodrugs in which the promoiety is attached to the secondary aniline group, bearing methylester protected (**24**) or free carboxylic acids (**25**).

**Scheme 3.** Synthesis of AMT prodrugs **24** and **25**[a]



[a]Reagents and conditions: (a) CbzCl, $NaHCO_3$, $H_2O$/dioxane, 21 °C, 2 h (89%). (b) $SOCl_2$, DMF, $CH_2Cl_2$, reflux, 24 h. (c) **9**·HCl, $Et_3N$, $CH_2Cl_2$, 21 °C, 2 h (>95%). (d) $H_2$, Pd/C, MeOH, 21°C, 12 h. (e) HCl in $Et_2O$ (>95%). (f) $NaBH_4$, MeOH, 21 °C, 24 h (67%). (g) DMA, 55 °C, 3 days (43%). (h) NaOH, $H_2O$, 21°C, 5 min (40%).

**Activation of prodrugs.** For a proof-of-principle of the prodrug strategy, a study on the activation of **11–13**, **17**, **24**, and **25** under patho-physiological concentrations of $H_2O_2$ was performed. The experiment consisted on the incubation of test compounds in a 30% DMSO in PBS mixture at a concentration of 50 μM and $H_2O_2$ concentration found in inflammatory tissue (0.5 mM, 10 equiv.) at 37 °C. The activation was followed by RP-UPLC-MS (see Figure S1 and Figure S2 for examples on prodrug **17** and **25** chromatograms, respectively). Negative controls (no $H_2O_2$ addition) were performed in parallel (Figure S3). The rationale behind the substitution of a hydrogen atom in the *ortho*-position of the phenylboronic acid promoiety by a methyl group or a fluorine atom was the reactivity towards oxidative deboronation. Interestingly, prodrugs **11–13** were activated and released **10** within less than 3 h (>95% conversion by UPLC-MS), therefore no effects on the reaction rate by *ortho*-substituents was observed under the conditions tested (Figure 2, left). Furthermore, **17** was activated within the same time frame, releasing exclusively MTX, with no significant difference compared to **11–13**. The plot of the formation of prodrug activation products *versus* time can be found in the SI (Figure S4). Moreover, a concentration dependent release of drug was observed as determined in an activation assay using different concentrations of $H_2O_2$ (see Figure S5).

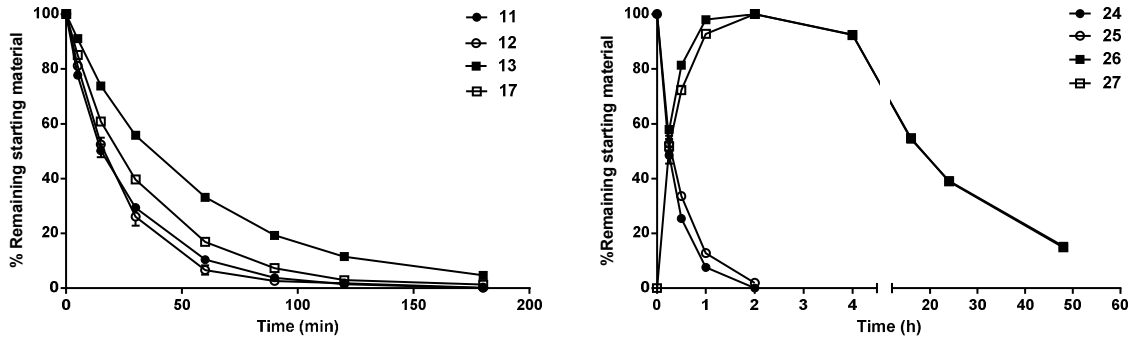
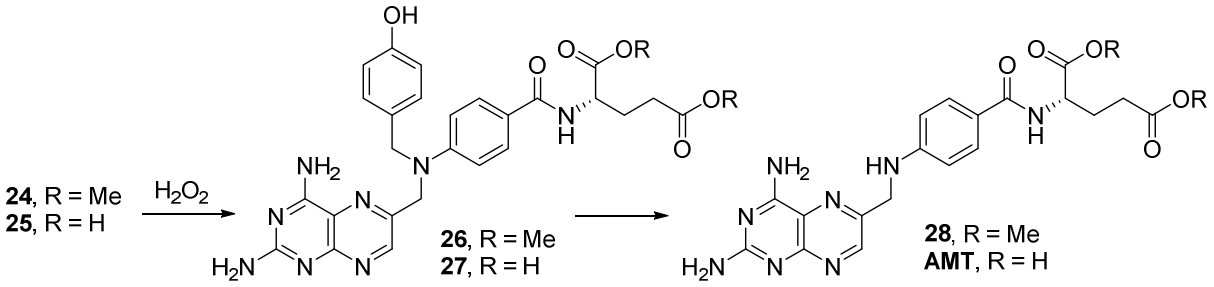



**Figure 2.** Activation of MTX prodrugs **11–13** and **17** (left), and AMT-prodrugs **24** and **25**, as well as formation and disappearance of intermediates **26** and **27** (right). The experiment was carried out at a compound concentration of 50 μM and 0.5 mM $H_2O_2$ (10 equiv.) in a mixture of 30% DMSO in PBS at 37 °C. Hidden error bars are smaller than symbols.

The activation of AMT prodrugs **24** and **25** was also examined. Full consumption of prodrugs was observed in a similar timeframe compared to the MTX prodrugs (Figure 2, right). AMT prodrugs did not release AMT nor **28** within the first two hours of incubation, but instead afforded the corresponding phenol intermediates **26** and **27** (Scheme 4). After 2 h of assay initiation, full disappearance of the prodrugs was observed and **26** and **27** started to slowly release AMT and **28**, in a $H_2O_2$ concentration independent manner as studied in a separate assay (see Figure S5). Full formation of products was not observed even after 48 h of incubation. The formation of the relatively stable intermediates **26** and **27** could potentially allow them to disperse from the inflammatory environment and exert non-selective drug actions upon AMT release, unlike for the MTX-prodrugs **11–13** and **17**, where phenol intermediates were not observed due to the faster self-immolation. This will have to be further evaluated in a future study of the AMT prodrugs.

**Scheme 4.** Activation of AMT prodrugs **24** and **25** with $H_2O_2$ to form the intermediates **26** and **27**, respectively, which slowly release **28** and AMT.




**Kinetic solubility.** The low solubility of **11–13** and **24** in the $H_2O_2$ activation assay required the use of high concentrations of DMSO (30%) in PBS. Therefore, the evaluation of the prodrug solubility was prioritized in the development program. Kinetic solubility in PBS was measured at 37 $^{o}$C and the results were compared to those determined for the parent drugs, showing low solubility (<10 μg/mL) for the methyl ester prodrugs **11–13** and **24**, according to the guidelines for oral administration in humans and animal dosing formulations (Table 1). On the other hand, prodrugs **17** and **25** showed moderate to good solubility for predicted oral dosing in humans (55 and 48 μg/mL, respectively), although still low for animal dosing applications. The low solubility of **11–13** and **24** prompted the decision to select **17** and **25** for further *in vitro* studies.

**Table 1.** Kinetic solubility of **11**, **12**, **13**, **17**, **24**, and **25**.

| **Prodrug** | **Kinetic solubility** | |
|---|---|---|
| | μM | μg/mL |
| **11** | 3.0 ± 0.5 | 2.0 ± 0.3 |
| **12** | 3.1 ± 1.4 | 2.1 ± 0.9 |
| **13** | 4.6 ± 0.9 | 3.1 ± 0.6 |
| **17** | 87 ± 2.3 | 55 ± 1.5 |
| **24** | 4.1 ± 0.2 | 2.5 ± 0.1 |
| **25** | 83 ± 0.6 | 48 ± 0.4 |
| **MTX** | 2137 ± 0.0 | 970 ± 0.0 |
| **AMT** | >1000 ± 0.0 | >440 ± 0.0 |

**Chemical (pH 7.4), SGF, SIF, plasma, and metabolic stability.** The stability of **17** and **25** was evaluated in different relevant physiological environments including chemical stability at blood pH 7.4 as well as in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), human and mouse plasma and liver microsomes. Firstly, the half-lives $t_{1/2}$ of **17** and **25** in PBS (pH 7.4) at 37 $^{o}$C were determined to 238 and 577 h, respectively, with stabilities higher than 90% after 24 h in both cases (Table 2 and Figure S6). Secondly, the stability in SGF and SIF was studied and half-lives calculated to 8 and 770 h in SGF, and 239 and 866 h in SIF for **17** and **25**, respectively (Table 2 and Figure S7). The shorter half-life of **17** in SGF was found to be pH-dependent rather than a result of enzymatic hydrolysis (Figure S7). Thirdly, the stability in human and mouse plasma proved to be high, with 70% and 82% of **17** and **25** remaining after 24 h in human plasma, respectively, and 62% and 91% after 8 h in mouse plasma (see Figure S8). Their half-lives were calculated using first order kinetics and the results are shown in Table 2. Lastly, the metabolic stability was evaluated by the determination of intrinsic clearance ($CL_{int}$) in human and mouse pooled liver microsomes. Intrinsic clearance of **17** and **25** was calculated to 22 and 1.2 μL/min/mg respectively in human microsomes, while 6.4 and 2.6 μL/min/mg in mouse microsomes (Table 2 and Figure S9), indicating their high metabolic stability.

**Table 2.** Chemical (pH 7.4), SGF, SIF, plasma, and metabolic stabilities of prodrug candidates **17** and **25**.

| | Chemical Stability (pH 7.4) | SGF Stability | SIF Stability | Human Plasma Stability | Mouse Plasma Stability | Human Microsomal Stability | Mouse Microsomal Stability |
|---|---|---|---|---|---|---|---|
| **Prodrug** | **$t_{1/2}$ (h)** | **$t_{1/2}$ (h)** | **$t_{1/2}$ (h)** | **$t_{1/2}$ (h)** | **$t_{1/2}$ (h)** | **$CL_{int}$ (μL/min/mg)** | **$CL_{int}$ (μL/min/mg)** |
| **17** | 238 | 8 | 239 | 70 | 17 | 2.2 | 6.4 |
| **25** | 577 | 770 | 866 | 56 | 63 | 1.2 | 2.6 |

**Anti-arthritic efficacy and preliminary toxicity.** The efficacy of the prodrug candidates **17** and **25** was evaluated in the collagen type-II induced arthritis (CIA) mouse model. This model has been found to be characterised by oxidative stress[58] and increased concentrations of $H_2O_2$ in the joint tissue, paws and limbs,[59,60] hence it represents a good model for the evaluation of the prodrug concept. Prior to the *in vivo* analysis, the prodrug concept was evaluated *in vitro* for **17** and **25**. To this end, the cell viability inhibition of the prodrugs against two MTX- and AMT-sensitive cancer cell lines, NCI-H460 (lung cancer) and MCF-7 (breast cancer),[61] was tested. The prodrugs showed significantly reduced efficacy in the two cell lines when applied directly to the cells as compared to when they were activated by pre-incubation with non-cytotoxic concentration of $H_2O_2$ (Figures S10–S12). Additionally, the $IC_{50}$ values of the pre-activated prodrugs **17** and **25** showed comparable biological activity to MTX and AMT (Figure S13 and S14) in the two selected cancer cell lines. A detailed description of the assays can be found in the SI.

The *in vivo* anti-arthritic efficacy and preliminary toxicity study was performed using a therapeutic intervention set up in the CIA model and the parent drugs MTX and AMT were included as treatment controls. Vehicle control (2% DMSO in PBS), MTX (7.0 mg/kg), and equimolar doses of AMT, **17**, and **25** were *i.p.* administered on a daily basis for two weeks, starting at onset of disease (day 27 after first immunization). Mean arthritis severity score and average animal body weight were measured three times per week. Reduced incidence of arthritis and reduction of macroscopic arthritis score was observed both for the drugs and prodrugs in comparison to the control group (vehicle) (Figure 3, left). Additionally, remission of arthritis started to be detected at the end of the study for MTX, **17**, and **25**, with no statistically significant difference in efficacy between them. Interestingly, the AMT-group had to be sacrificed at day 33

due to significant toxicity measured as decreased average body weight in the group, while the AMT prodrug **25** did not show any sign of side-effects (Figure 3, right). Progressive weight loss was observed in the MTX-group towards the end of the study but not in the group treated with **17**. These results indicate the great potential of **17** and **25** as prodrugs of MTX and AMT, respectively, showing comparable efficacy in managing arthritis development and control of disease activity, and reducing side-effects compared to the parent drugs.

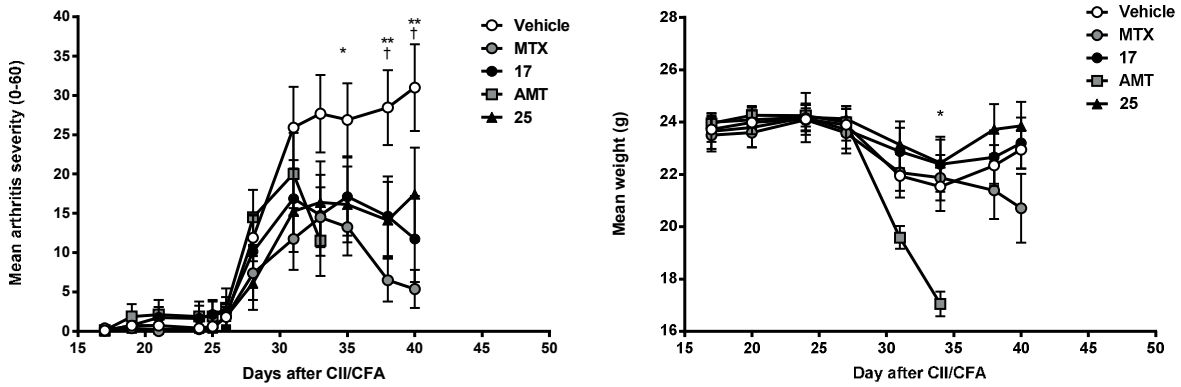



**Figure 3.** Suppression of CIA development in DBA/1J mice (left) after treatment with MTX (7 mg/kg) and equimolar amounts of AMT (6.8 mg/kg), **17** (9.7 mg/kg), and **25** (8.8 mg/kg) (n = 8 per group). Mice were given the indicated dose of test compound once per day, starting on day 27, and arthritis scoring was performed three times per week. Data represents mean values of arthritic score ± SEM. Statistics were calculated using a one-tailed non parametric Mann-Whitney. * $p < 0.05$ and ** $p < 0.01$ between MTX and vehicle and † $p < 0.05$ between **17** and vehicle. General health of mice (right) evaluated three times per week as the average body weight in groups of animals (n = 8) tested with vehicle, MTX, AMT, **17**, and **25**. Data represents mean values of bodyweight ± SEM. * $p < 0.05$ between AMT and vehicle. One animal in the vehicle and the **25** groups were sacrificed pre-termination due to high arthritis scores. The AMT group was removed at day 33 due to decline in health. AMT, aminopterin; CIA, collagen induced-arthritis; CII, collagen type-II; MTX, methotrexate.

Although no toxicity associated to prodrugs **17** and **25** has been observed in the animal assays performed, it is of relevance for future studies of the potential toxicities to also account for the release of quinone methides as prodrug activation end-product. Their reactivity and toxicity has been linked and reviewed elsewhere,[62] and prior studies have demonstrated that it is possible to modulate these factors through judicious choice of the substitution pattern on the quinone

methides released. Noteworthy, quinone moieties are known in long-term use marketed drugs,[62] and newly developed ROS-sensitive drug delivery systems which employ phenyl boronates as the responsive chemical entity have shown good safety profiles after long-term administration in mice.[63]

## CONCLUSION

In this report, we have described the synthesis of new arylboronic acid-based hydrogen peroxide-sensitive prodrugs of MTX and AMT for site-selective delivery of drugs to inflammatory tissues associated to RA with the aim of reducing side effects in MTX and AMT RA therapy. Among the set of prodrugs synthesized, **17** and **25** showed moderate to good solubility, high chemical stability, high stability in simulated gastrointestinal fluids, and high plasma and metabolic stabilities both in human and mouse plasma and liver microsomes. Their comparable efficacy and lower toxicity in comparison to parental drugs was identified using a CIA mouse model of RA. Future work will focus on additional *in vivo* studies to establish optimal dosing and to map toxicity in more detail to hopefully advance these promising prodrugs into clinical evaluations.

## EXPERIMENTAL SECTION

### General considerations

Unless otherwise stated, commercially available reagents were used without further purification and all solvents were of HPLC quality. Reactions under nitrogen atmosphere were performed in oven- or flame-dried glassware and anhydrous solvents. Anhydrous $CH_2Cl_2$, $CH_3CN$, THF, DMF, and toluene were obtained from Innovative Technology PS-MD-7 Pure

solve solvent purification system. All reactions were monitored by thin-layer chromatography (TLC) and/or reversed-phased ultra-performance liquid chromatography mass spectrometry (RP-UPLC-MS). Analytical TLC was conducted on Merck aluminium sheets covered with silica (C60). The plates were either visualized under UV-light or stained by dipping in a developing agent followed by heating. $KMnO_4$ (3 g in water (300 mL) along with $K_2CO_3$ (20 g) and 5% aqueous NaOH (5 mL)) or Ninhydrin (3 g in a mixture of *n*-butanol (200 mL) and AcOH (6 mL)) were used as developing agents. Flash column chromatography was performed using Matrex 60 Å, 35–70 μ silica gel. All new compounds were characterized by IR, $^1$H NMR, $^{13}$C NMR, HRMS (ESI) and melting point (m.p.) when applicable. For recording of $^1$H NMR and $^{13}$C NMR a Bruker Ascend with a Prodigy cryoprobe (operating at 400 MHz for proton and 101 MHz for carbon) was used. Unless otherwise stated, all NMR spectra were recorded at 25 $^o$C. The chemical shifts (δ) are reported in parts per million (ppm) and the coupling constants (*J*) in Hz. For spectra recorded in $CDCl_3$, signal positions were measured relative to the signal for $CHCl_3$ (7.26 ppm for $^1$H NMR and 77.16 ppm for $^{13}$C NMR). For spectra recorded in DMSO-$d_6$ signal positions were measured relative to the signal for DMSO (δ 2.50 ppm for $^1$H NMR and 39.52 ppm for $^{13}$C NMR). For spectra recorded in $D_2O$ signal positions were measured relative to the signal for $H_2O$ (δ 4.79 ppm for $^1$H NMR at 25 $^o$C).[64] The $^{13}$C NMR signal of the *ipso* carbon to the boron atom was not observed in any characterized compound, aulthough they were identified by the characteristic HMBC correlation signals. Infrared (IR) spectra were recorded on a Bruker Alpha-P FT-IR (Bruker) instrument. Neat application into the apparatus was used for all compounds. Transmittance units were plotted in the y axis and wavenumber ($cm^{-1}$) on the x axis. Optical rotations were measured on a Perkin Elmer Model 241 Polarimeter (cuvette 1.0 mL, 100 mm) using a sodium source lamp (589 nm, 20 $^o$C). Melting points (m.p.) were recorded

using a Stuart melting point SMP30, and reported in $^{o}$C, uncorrected. Analytical RP-UPLC-MS (ESI) analysis was performed on a Waters AQUITY RP-UPLC system equipped with a diode array detector using a Thermo accucore C18 column (d 2.6 μm, 2.1 x 50 mm; column temp: 50 °C; flow: 0.6 mL/min). Four different methods were use. Method A: eluents A (0.1% $HCO_2H$ in milli-Q water) and B (0.1% $HCO_2H$ in $CH_3CN$) were used in a linear gradient (5% B to 100% B) in a total run time of 2.6 min. Method B: eluents A (0.1% $HCO_2H$ in $H_2O$) and B (0.1% $HCO_2H$ in $CH_3CN$) were used in a linear gradient (5% B to 100% B) in a total run time of 5.0 min. Method C: eluents A (10 mM $NH_4OAc$ in milli-Q water) and B (0.1% $NH_4OAc$ in milli-Q water/MeCN, 90/10, v/v) were used in a linear gradient (5% B to 100% B) in a total run time of 2.6 min. Method D: eluents A (0.1% $NH_4OAc$ in $H_2O$) and B (0.1% $NH_4OAc$ in $CH_3CN$) were used in a linear gradient (5% B to 100% B) in a total run time of 5.0 min. The LC system was coupled to a SQD mass spectrometer operating in both positive and negative electrospray modes. The temperature for all recordings was 20 °C. Analytical LC-HRMS (ESI) analysis was performed on an Agilent 1100 RP-LC system equipped with a diode array detector using a Phenomenex Luna C18 column (*d* 3 μm, 2.1 x 50 mm; column temp: 40 °C; flow: 0.4 mL/min). Eluents A (0.1% $HCO_2H$ in $H_2O$) and B (0.1% $HCO_2H$ in $CH_3CN$) were used in a linear gradient (20% B to 100% B) in a total run time of 15 min. The LC system was coupled to a Micromass LCT orthogonal time-of-flight mass spectrometer equipped with a Lock Mass probe operating in positive or negative electrospray mode. Purification of reactions by preparative RP-HPLC was performed on a Waters Alliance reverse-phase HPLC system consisting of a Waters 2545 Binary Gradient Module equipped with either an xBridge BEH C18 OBD Prep Column (130 Å, 5 μm, 30 x 150 mm) or an xBridge Peptide BEH C18 OBD Prep Column (130 Å, 5 μm, 19 mm x 100 mm) both operating at 20 °C and a flow rate of 20 mL/min, a Waters Photodiode Array Detector

(detecting at 210–600 nm), a Waters UV Fraction Manager, and a Waters 2767 Sample Manager. Elution was carried out in a reversed-phase gradient fashion combining A1 (0.1% $HCO_2H$ in milli-Q water) and B1 (0.1% $HCO_2H$ in $CH_3CN$) or A2 (5 mM $NH_4OAc$ in $H_2O$) and B2 (5 mM $NH_4OAc$ in $CH_3CN$): 5% B to 70 % B in 10 min, hold for 3.5 min, then 70% B to 100% B in 1.5 min, and hold 3 minutes. Total run time: 20 min. The purity of the compounds was assessed by RP-UPLC-UV and NMR, and purities ≥95% were considered acceptable for evaluation purposes both *in vitro* and *in vivo* assays.

**Synthesis**

**3-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonochloridate (4b).** *General procedure A:* a suspension of **3b** (0.9 g, 5.4 mmol) and pinacol (0.7 g, 6.0 mmol) in anhydrous THF (25 mL) was refluxed for 16 h under an $N_2$ atmosphere. The solids dissolved during the reaction time. Then, the mixture was concentrated *in vacuo* and the residue purified by flash column chromatography on silica gel using a mixture of $CH_2Cl_2$/EtOAc (9/1, v/v) as the eluent to give the title compound **4b** as a clear oil (1.27 g, 95%) which solidified upon storage in the refrigerator. $R_f$ = 0.84 (silica, $CH_2Cl_2$/EtOAc, 1/1, v/v); m.p.: 38.5 – 41.3 $^oC$; IR (neat, $cm^{-1}$): 3390.6, 2978.1, 2925.3, 2867.7, 1609.0, 1347.1, 335.9; $^1H$ NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.4 Hz, 1H), 7.17 – 7.09 (m, 2H), 4.67 (s, 2H), 2.54 (s, 3H), 1.34 (s, 12H); $^{13}C$ NMR (101 MHz, $CDCl_3$) δ 145.5, 143.6, 136.4, 128.3, 123.2, 83.6, 65.4, 25.0, 22.3; HRMS (ESI) *m/z*: calcd for $C_{14}H_{22}BO_3$ $[M+Na]^+$ 271.1476, found 271.1512.

**(3-Fluoro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanol (4c).** Following *general procedure A* using **3c** (0.90 g, 5.30 mmol), the title compound **4c** was afforded as a clear oil (1.26 g, 94%) after purification by flash column chromatography on silica gel using a mixture

of $CH_2Cl_2$/EtOAc (9/1, v/v) as the eluent. The oil became a white solid upon storage in the refrigerator. $R_f$ = 0.71 (silica, eluent $CH_2Cl_2$/EtOAc, 1/1, v/v); m.p.: 52.5 – 56.4 °C (Litt.[65] 35–38); IR (neat, $cm^{-1}$): 3424.6, 2978.6, 2932.3, 2864.3, 1624.1, 1351.8; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.77 – 7.67 (m, 1H), 7.12 (d, *J* = 7.6 Hz, 1H), 7.06 (d, *J* = 10.1 Hz, 1H), 4.72 (s, 2H), 1.36 (s, 12H);[65] $^{13}$C NMR (101 MHz, $CDCl_3$) δ 167.6 (d, *J* = 251.4 Hz), 147.2 (d, *J* = 8.0 Hz), 137.2 (d, *J* = 8.4 Hz), 121.7 (d, *J* = 2.9 Hz), 113.4 (d, *J* = 24.7 Hz), 84.0, 64.6, 25.0.[65]

**4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonochloridate (5a).** *General procedure B:* a solution of 4**a** (2.0 g, 8.54 mmol) in anhydrous dioxane (25 mL) was treated with phosgene (20% in toluene, 22.6 mL, 42.7 mmol) and the reaction was stirred for 20 h under an $N_2$ atmosphere at 21 °C. The mixture was concentrated *in vacuo*, redissolved and concentrated with toluene (2x) to afford the crude title compound **5a** as a clear oil (2.5 g, > 95%). The crude chloroformate was used in subsequent reactions within few hours after its isolation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.1 Hz, 2H), 7.38 (d, *J* = 8.1 Hz, 2H), 5.31 (s, 2H), 1.35 (s, 12H);[66] $^{13}$C NMR (101 MHz, $CDCl_3$) δ 150.8, 136.2, 135.4, 128.0, 84.2, 73.4, 25.0.[66]

**3-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonochloridate (5b).** Following *general procedure B* using **4b** (1.2 g, 4.8 mmol), the title compound **5b** was afforded as a clear oil (1.5 g, >95%). The crude chloroformate was used in subsequent reactions within few hours after its isolation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.0 Hz, 1H), 7.20 – 7.13 (m, 2H), 5.26 (s, 2H), 2.55 (s, 3H), 1.34 (s, 12H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 150.8, 145.7, 136.5, 135.7, 130.1, 125.0, 83.8, 73.5, 25.0, 22.3.

**3-Fluoro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonochloridate (5c).** Following *general procedure B* using **4c** (1.2 g, 4.8 mmol), the crude product **5c** was afforded as

a white solid (1.5 g, >95%). The crude chloroformate was used in subsequent reactions within few hours after isolation. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.77 (dd, *J* = 7.5, 6.1 Hz, 1H), 7.15 (dd, *J* = 7.5, 1.3 Hz, 1H), 7.07 (dd, *J* = 9.6, 1.1 Hz, 1H), 5.29 (s, 2H), 1.36 (s, 12H);[65] $^{13}$C NMR (101 MHz, $CDCl_3$) δ 167.7 (d, *J* = 251 Hz), 150.8, 138.92 (d, *J* = 8.3 Hz), 137.6 (d, *J* = 8.5 Hz), 123.5 (d, *J* = 3.2 Hz), 115.3 (d, *J* = 25.4 Hz), 84.3 (s), 72.2 (s), 25.0 (s).[65]

**4-(((2,4-Diaminopteridin-6-yl)methyl)(methyl)amino)benzoic acid (7).** 2,4-Diamino-6-(hydroxymethyl)pteridine hydrochloride **6**·HCl (4.4 g, 19 mmol) was dissolved in hot water (150 mL) and after cooling to 21 °C the solution was neutralized with 1 M aq. NaOH to pH ~ 7 (ca. 20 mL). The formed precipitates were collected by filtration, washed with water, and dried *in vacuo* over $P_2O_5$ to afford an orange-beige solid corresponding to 2,4-diamino-6-(hydroxymethyl)pteridine. The solid was suspended in anhydrous DMA (25 mL) and triphenylphosphine dibromide (18 g, 43 mmol) was added to the suspension. The turbid and dark mixture was stirred for 24 h under an $N_2$ atmosphere at 20 °C. Then, 4-aminobenzoic acid (3.0 g, 20 mmol) was added to the reaction and stirred for an additional three days. The reaction mixture was poured into 250 mL of 0.33N NaOH and the precipitate was filtered off. The filtrate was neutralized with 10% aq. acetic acid (ca. 20 mL) and the precipitate formed upon neutralization was filtered off, washed with water, triturated over MeOH, filtered, and dried *in vacuo* to afford the title compound **7** as an orange-beige solid (5.70 g, 91%). m.p.: > 240 °C (decomp.) (lit.[52] 242 °C); IR (neat, $cm^{-1}$): 3378.7, 3279.5, 2233.3, 1663.6, 1598.6, 1288.4, 1186.0;[67] $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 12.15 (br s, 1H), 8.63 (s, 1H), 8.17 (br s, 1H), 7.94 (br s, 1H), 7.73 (d, *J* = 9.0 Hz, 2H), 7.04 (br s, 2H), 6.83 (d, *J* = 9.0 Hz, 2H), 4.81 (s, 2H), 3.23 (s, 3H);[26] $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 167.8, 163.2, 161.1, 152.3, 149.5, 147.8, 131.5, 122.2, 118.1, 111.7, 100.0, 55.2, 39.6.

**Dimethyl L-glutamate hydrochloride (9·HCl).** Thionyl chloride (5.0 mL, 68 mmol) was added dropwise to 50 mL of anhydrous MeOH at 0 °C. The mixture was stirred for 30 min at the same temperature followed by addition of L-glutamic acid **8** (5.0 g, 34 mmol). Then, the reaction was stirred under an $N_2$ atmosphere for 3 days at 21 °C and concentrated *in vacuo* to afford the title compound **9**·HCl as a colourless solid (7.19 g, >95%). The crude product was used without further purification in the next reactions. m.p.: 63.2 – 64.0 °C (lit.[68] 77–79); $[\alpha]_D^{20}$ = +25.0 (c 5.0, $H_2O$)[69]; IR (neat, $cm^{-1}$): 2952.45, 2902.73, 2852.33, 1728.67[70]; $^1H$ NMR (400 MHz, $D_2O$) δ 4.24 – 4.19 (m, 1H), 3.85 (s, 3H), 3.73 (s, 3H), 2.65 (td, *J* = 7.3, 2.2 Hz, 2H), 2.37 – 2.13 (m, 2H);[70] $^{13}C$ NMR (101 MHz, $D_2O$) δ 174.8, 170.2, 53.6, 52.4, 52.0, 29.2, 24.7.[70]

**Dimethyl (4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (10).** To a solution of **7** (3.0 g, 9.2 mmol) in anhydrous DMF (80 mL) was added $Et_3N$ (6.4 mL, 46.1 mmol) followed by PyBOP (6.5 g, 12.4 mmol). The mixture was stirred for 30 min under an $N_2$ atmosphere at 21 °C. Then, **9**·HCl (2.1 g, 9.9 mmol) was added and the reaction mixture was stirred for 5 h at the same temperature. Then, the reaction was filtered through a path of Celite® and the filtrate concentrated *in vacuo*. Next, the residue was dissolved in a mixture of EtOAc/$CHCl_3$ (150 mL, 1/1) and poured into 750 mL of $Et_2O$ at 0 °C under strong stirring. The resultant suspension was filtered off, washed with $Et_2O$ and cold water, triturated in hot MeOH, and filtered off again. The resultant orange solid wash purified by flash column chromatography on silica gel using a mixture of $CH_2Cl_2$/MeOH (92.5/7.5, v/v) as the eluent to give the title compound **10** as a yellow solid (2.39 g, 54%). $R_f$ = 0.48 (silica, MeOH/$CH_2Cl_2$, 1/9, v/v); m.p.: >168.6 (decomp.) (lit.[52] 130–134); $[\alpha]_D^{20}$ = +2.6 (c 0.5, DMSO); IR (neat, $cm^{-1}$): 3306.96, 3128.91, 2950.99, 2923.52, 1731.36, 1626.09, 1604.36, 1503.14, 1435.41; $^1H$ NMR (400 MHz, DMSO-$d_6$) δ 8.56 (s, 1H), 8.34 (d, *J* = 7.4 Hz, 1H), 7.77 – 7.69 (m, 3H), 7.45 (br s, 1H), 6.82 (d,

*J* = 8.8 Hz, 2H), 6.61 (br s, 2H), 4.78 (s, 2H), 4.39 (ddd, *J* = 9.4, 7.4, 5.6 Hz, 1H), 3.61 (s, 3H), 3.57 (s, 3H), 3.21 (s, 3H), 2.41 (t, *J* = 7.4 Hz, 2H), 2.17 –1.72 (m, 2H);[71] $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.7, 172.6, 166.4, 162.9, 162.7, 155.2, 151.0, 149.2, 145.9, 129.0, 121.4, 120.7, 111.0, 54.9, 51.8, 51.7, 51.4, 38.7, 30.0, 25.8;[71] HRMS (ESI) *m/z*: calcd for $C_{22}H_{27}N_8O_5$ $[M+H]^+$ 483.2099, found 483.2119.

**(*S*)-(4-((((2-Amino-6-(((4-((1,5-dimethoxy-1,5-dioxopentan-2-yl)carbamoyl)phenyl)(methyl)amino)methyl)pteridin-4-yl)carbamoyl)oxy)methyl) phenyl)boronic acid (11).** *General procedure C:* to a suspension of **10** (313 mg, 0.68 mmol) in anhydrous $CH_2Cl_2$ (25 mL) was added DMAP (417 mg, 3.42 mmol) followed by DIPEA (0.60 mL, 3.42 mmol). The mixture was cooled to 0 $^oC$ and a solution of **5a** (1.01 g, 3.42 mmol) in $CH_2Cl_2$ (10 mL) was added dropwise. The reaction was allowed to warm to 21 $^oC$ and stirred for 6 h under an $N_2$ atmosphere. Then, the mixture was diluted with $CH_2Cl_2$ (100 mL), washed with 0.1 M aq. HCl (2 x 75 mL), sat. aq. $NaHCO_3$ (2 x 75 mL), and brine (75 mL). The organic layer was dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford a yellow solid that was purified by reversed-phase preparative HPLC. The $CH_3CN/H_2O$ collected fractions containing the pinacolate intermediate were combined into a 250 mL round bottom flask and HCl cc. (0.3 mL, pH ~ 2) was added. The reaction mixture was stirred for 16 h at 21 $^oC$ and quenched with sat. aq. $NaHCO_3$ (ca. 50 mL). After removal of the $CH_3CN$ under rotatory evaporation the formed precipitate was filtered off, washed with $H_2O$ and dried *in vacuo* to afford the title compound **11** as a yellow solid (122 mg, 27%). Additional preparative HPLC purification was sometimes needed in specific substrates. m.p.: >153.0 $^oC$ (decomp.); $[\alpha]_D^{20}$ = +4.7 (c 0.32, DMSO); IR (neat, $cm^{-1}$): 3354.4, 3233.1, 2954.7, 1732.5, 1627.5, 1605.2, 1573.9, 1536.9, 1198.5, 1173.2; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.88 (br s, 1H), 8.68 (s, 1H), 8.34 (d, *J* = 7.5

Hz, 1H), 8.08 (s, 2H), 7.82 (d, *J* = 7.8 Hz, 2H), 7.72 (d, *J* = 8.8 Hz, 2H), 7.43 (d, *J* = 7.8 Hz, 2H), 7.27 (br s, 2H), 6.82 (d, *J* = 8.8 Hz, 2H), 5.24 (s, 2H), 4.84 (s, 2H), 4.39 (ddd, *J* = 9.3, 7.5, 5.7 Hz, 1H), 3.60 (s, 3H), 3.56 (s, 3H), 3.20 (s, 3H), 2.40 (t, *J* = 7.4 Hz, 2H), 2.15 – 1.90 (m, 2H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.7, 172.6, 166.4, 162.0, 157.8, 155.1, 151.1, 150.7, 150.3, 148.0, 137.7, 134.2, 129.0, 127.0, 120.9, 120.9, 111.1, 66.7, 54.9, 51.8, 51.7, 51.4, 38.9, 30.0, 25.8. HRMS (ESI) *m/z*: calcd for $C_{30}H_{34}BN_8O_9$ $[M+H]^+$ 661.2536, found 661.2566.

**(*S*)-(4-((((2-Amino-6-(((4-((1,5-dimethoxy-1,5-dioxopentan-2-yl)carbamoyl)phenyl)(methyl)amino)methyl)pteridin-4-yl)carbamoyl)oxy)methyl)-2-methylphenyl)boronic acid (12).** Following *general procedure C* using **5b** (1.45 g, 4.66 mmol), the title compound **12** was afforded as a yellow solid (179 mg, 22%). m.p.: >166.0 °C (decomp.); $[\alpha]_D^{20}$ = +4.0 (c 0.7, DMSO); IR (neat, cm$^{-1}$): 3340.8, 3230.2, 2951.6, 1741.8, 1604.5, 1510.3, 1197.7, 1166.6, 1028.4; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.82 (s, 1H), 8.69 (s, 1H), 8.34 (d, *J* = 7.5 Hz, 1H), 8.02 (s, 2H), 7.72 (d, *J* = 9.0 Hz, 2H), 7.46 (d, *J* = 8.0 Hz, 1H), 7.28 (br s, 2H), 7.23 – 7.16 (m, 2H), 6.83 (d, *J* = 9.0 Hz, 2H), 5.18 (s, 2H), 4.84 (s, 2H), 4.39 (ddd, *J* = 9.5, 7.5, 5.4 Hz, 1H), 3.61 (s, 3H), 3.56 (s, 3H), 3.20 (s, 3H), 2.44 – 2.37 (m, 5H), 2.13 – 1.88 (m, 2H).; $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.7, 172.6, 166.4, 162.0, 157.8, 155.1, 151.0, 150.7, 150.2, 147.9, 141.4, 136.2, 133.3, 129.0, 128.9, 124.3, 120.9, 120.8, 111.1, 66.7, 54.9, 51.8, 51.7, 51.3, 38.9, 29.9, 25.8, 22.1; HRMS (ESI) *m/z*: calcd for $C_{31}H_{36}BN_8O_9$ $[M+H]^+$ 675.2693, found 675.2720.

**(*S*)-(4-((((2-Amino-6-(((4-((1,5-dimethoxy-1,5-dioxopentan-2 yl)carbamoyl)phenyl)(methyl)amino)methyl)pteridin-4-yl)carbamoyl)oxy)methyl)-2-fluorophenyl)boronic acid (13).** Following *general procedure C* using **5c** (1.30 g, 4.19 mmol), the title compound **13** was afforded as a yellow solid (237 mg, 34%). m.p.: >158.1 °C

(decomp.); $[\alpha]_D^{20}$ = +4.3 (c 0.6, DMSO); IR (neat, $cm^{-1}$): 3499.4, 3448.3, 3360.5, 3326.1, 3218.7, 2952.9, 1760.0, 1733.0, 1625.6, 1490.3, 1196.3, 1171.6; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.95 (s, 1H), 8.69 (s, 1H), 8.34 (d, *J* = 7.5 Hz, 1H), 8.22 (s, 2H), 7.72 (d, *J* = 8.8 Hz, 2H), 7.59 (t, *J* = 7.0 Hz, 1H), 7.40 – 7.20 (m, 4H), 6.83 (d, *J* = 8.9 Hz, 2H), 5.25 (s, 2H), 4.85 (s, 2H), 4.39 (ddd, *J* = 9.4, 7.5, 5.7 Hz, 1H), 3.61 (s, 3H), 3.56 (s, 3H), 3.20 (s, 3H), 2.41 (t, *J* = 7.4 Hz, 2H), 2.16 – 1.86 (m, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 173.2, 173.04, 166.9, 165.9 (d, *J* = 244.5 Hz), 162.5, 158.3, 155.6, 151.5, 151.1, 150.7, 148.4, 140.6 (d, *J* = 8.3 Hz), 136.0(d, *J* = 9.8 Hz), 129.5, 123.4 (d, *J* = 2.7 Hz), 121.4, 121.3, 114.5 (d, *J* = 25.5 Hz), 111.6, 66.2, 55.4, 52.3, 52.2, 51.8, 39.1, 30.4, 26.2; HRMS (ESI) *m/z*: calcd for $C_{30}H_{33}BFN_8O_9$ $[M+H]^+$ 679.2442, found 679.2455.

**Bis(4-methoxybenzyl) (4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (14).** A solution of methotrexate (1.0 g, 2.20 mmol) in anhydrous DMF (50 mL) was treated with 1,1,3,3-tetramethylguanidine (0.55 mL, 4.4 mmol) at 0 °C and the mixture was stirred for 30 min under an $N_2$ atmosphere. Then, 4-methoxybenzyl chloride (0.59 mL, 4.4 mmol) was added dropwise and the mixture was allowed to warm to 21 °C and stirred for 24 h. Next, volatiles were removed *in vacuo* and the crude was purified by flash column chromatography on silica gel using a mixture of $CH_2Cl_2$/MeOH (gradient elution from 97/3 to 94/6, v/v) as the eluent to give the title compound **154**as a yellow solid (915 mg, 60%). $R_f$ = 0.24 (silica, $CH_2Cl_2$/MeOH, 95/5, v/v); m.p.: 96.5 – 99.2 °C; $[\alpha]_D^{20}$ = +8.0 (c 1.0, DMSO); IR (neat, $cm^{-1}$): 3325.0, 3181.8, 2936.0, 2835.6, 1730.6, 1603.3, 1511.2, 1442.8; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.56 (s, 1H), 8.34 (d, *J* = 7.5 Hz, 1H), 7.71 (d, *J* = 8.9 Hz, 2H), 7.65 (br s, 1H), 7.43 (br s, 1H), 7.27 (d, *J* = 6.9 Hz, 2H), 7.25 (d, *J* = 6.9 Hz, 2H), 6.90 – 6.86 (m, 4H), 6.82 (d, *J* = 8.9 Hz, 2H), 6.60 (br s, 2H), 5.10 – 4.99 (m, 2H), 4.97 (s, 2H), 4.78 (s, 2H), 4.42 (ddd, *J* = 9.6, 7.5,

5.4 Hz, 1H), 3.73 (s, 6H), 3.21 (s, 3H), 2.42 (t, *J* = 7.6 Hz, 2H), 2.13 – 1.90 (m, 2H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.1, 172.0, 166.5, 162.9, 162.7, 159.1, 155.2, 151.0, 149.2, 145.9, 129.8, 129.7, 129.0, 128.0, 127.9, 121.4, 120.8, 113.8, 111.0, 65.7, 65.3, 55.1, 54.8, 51.9, 38.8, 30.2, 25.8; HRMS (ESI) *m/z*: calcd for $C_{36}H_{39}N_8O_7$ $[M+H]^+$ 695.2936, found 695.2954.

**Bis(4-methoxybenzyl) (4-(((2-amino-4-((((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)carbonyl)amino)pteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamate (15).** To a suspension of **15** (800 mg, 1.15 mmol) in anhydrous $CH_2Cl_2$ (25 mL) was added DMAP (703 mg, 5.76 mmol) followed by DIPEA (1.0 mL, 5.76 mmol). The mixture was cooled to 0 °C and a solution of **5a** (1.71 g, 5.76 mmol) in $CH_2Cl_2$ (10 mL) was added dropwise. The reaction was allowed to warm to 21 °C and stirred for 5 h under an $N_2$ atmosphere. The mixture was then diluted with $CH_2Cl_2$ (100 mL), washed with 1 M aq. HCl (2 x 75 mL), sat. aq. $NaHCO_3$ (2 x 75 mL), and brine (75 mL). The organic layer was dried over $Na_2SO_4$, filtered and concentrated *in vacuo*. Purification of the residue by reversed-phase preparative HPLC afforded the title compound **15** as a yellow solid (800 mg, 73%). m.p.: >105.0 (decomp.); $[\alpha]_D^{20}$ = +8.8 (c 0.42, DMSO); IR (neat, cm$^{-1}$): 3351.5, 3230.0, 2975.5, 2836.5, 1731.3, 1605.6, 1512.6, 1356.9; $^1$H NMR (400 MHz, DMSO-$d_6$) 9.87 (s, 1H), 8.69 (s, 1H), 8.34 (d, *J* = 7.5 Hz, 1H), 7.72 (d, *J* = 7.9 Hz, 4H), 7.47 (d, *J* = 7.9 Hz, 2H), 7.33 – 7.17 (m, 6H), 6.91 – 6.85 (m, 4H), 6.82 (d, *J* = 9.0 Hz, 2H), 5.26 (s, 2H), 5.06 – 4.98 (m, 2H), 4.96 (s, 2H), 4.84 (s, 2H), 4.43 (ddd, *J* = 9.5, 7.4, 5.4 Hz, 1H), 3.72 (s, 6H), 3.19 (s, 3H), 2.41 (t, *J* = 7.4 Hz, 2H), 2.13 – 1.91 (m, 2H), 1.29 (s, 12H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.1, 172.0, 166.5, 162.0, 159.1 (2C), 157.8, 155.1, 151.1, 150.7, 150.3, 148.0, 139.3, 134.6, 129.8, 129.7, 129.0, 128.0, 127.9, 127.3, 121.0, 120.8, 113.8 (2C), 111.1, 83.7, 66.5, 65.7, 65.3, 55.1 (2C), 54.9, 51.9, 39.5, 30.2, 25.8, 24.7; HRMS (ESI) *m/z*: calcd for $C_{50}H_{56}BN_8O_{11}$ $[M+H]^+$ 955.4156, found 955.4191.

**(*S*)-(4-((((2-Amino-6-(((4-((1,5-bis((4-methoxybenzyl)oxy)-1,5-dioxopentan-2-yl)carbamoyl)phenyl)(methyl)amino)methyl)pteridin-4-yl)carbamoyl)oxy)methyl)phenyl)boronic acid (16).** To a solution of **15** (0.70 g, 0.73 mmol) in a mixture of $CH_3CN/THF/H_2O$ (200 mL, 2/1/1, v/v) was added cc. HCl until pH ~ 2. The reaction mixture was stirred at 21 $^oC$ for 6 h followed by neutralization with sat. aq. $NaHCO_3$ (approx. 50 mL). After removal of organic solvents *in vacuo*, the formed precipitate was filtered off, washed with $H_2O$, and purified by reversed-phase preparative HPLC to afford the title compound **16** as a yellow solid (199 mg, 31%). m.p.: >147.8 $^oC$; $[\alpha]_D^{20}$ = +8.3 (c 0.99, DMSO); IR (neat, $cm^{-1}$): 3362.3, 3230.6, 2954.1, 2836.7, 1732.0, 1628.9, 1605.5, 1512.4, 1198.7, 1170.9; $^1H$ NMR (400 MHz, DMSO-$d_6$) δ 9.10 (br s, 1H), 8.64 (br s, 1H), 8.49 (s, 2H), 8.35 (d, *J* = 7.5 Hz, 1H), 7.78 (d, *J* = 7.9 Hz, 2H), 7.71 (d, *J* = 8.9 Hz, 2H), 7.40 (d, *J* = 7.9 Hz, 2H), 7.37 – 7.10 (m, 6H), 6.87 (d, *J* = 8.7 Hz, 2H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.82 (d, *J* = 9.0 Hz, 1H), 5.21 (s, 2H), 5.10 – 4.98 (m, 2H), 4.96 (s, 2H), 4.83 (s, 2H), 4.42 (ddd, *J* = 9.6, 7.4, 5.4 Hz, 1H), 3.72 (s, 6H), 3.19 (s, 3H), 2.41 (t, *J* = 7.6 Hz, 2H), 2.15 – 1.79 (m, 2H); $^{13}C$ NMR (101 MHz, DMSO-$d_6$) δ 172.2, 172.0, 166.6, 162.1, 159.1 (2C), 157.8, 155.2, 151.1, 150.7, 150.3, 148.0, 132.7, 134.3, 129.9, 129.7, 129.1, 128.0, 127.9, 127.1, 121.0, 120.9, 113.8 (2C), 111.1, 66.7, 65.8, 65.4, 55.1 (2C), 54.9, 51.9, 39.35, 30.2, 25.8; HRMS (ESI) *m/z*: calcd for $C_{44}H_{46}BN_8O_{11}$ $[M+H]^+$ 873.3374, found 873.3405.

**(4-(((2-amino-4-((((4-Boronobenzyl)oxy)carbonyl)amino)pteridin-6-yl)methyl)(methyl)amino)benzoyl)-L-glutamic acid (17).** A solution of **16** (150 mg, 0.17mmol) in a mixture of 5% TFA in anhydrous $CH_2Cl_2$ (16 mL) was stirred for 25 min under an $N_2$ atmosphere at 21 $^oC$. The crude mixture was then concentrated *in vacuo* and the residue purified by reversed-phase preparative HPLC to afford the title compound **17** as an yellow/orange solid

(59 mg, 54%). m.p.: >200 °C (decomp.); $[\alpha]_D^{20}$ = +10.0 (c 0.50, DMSO); IR (neat, $cm^{-1}$): 3336.8, 3203.3, 2929.5, 1728.8, 1604.5, 1505.5, 1199.4, 1171.1; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.29 (br s, 2H), 9.89 (s, 1H), 8.68 (s, 1H), 8.20 (d, *J* = 7.8 Hz, 1H), 8.07 (s, 2H), 7.82 (d, *J* = 7.9 Hz, 2H), 7.73 (d, *J* = 8.9 Hz, 2H), 7.43 (d, *J* = 7.9 Hz, 2H), 7.28 (br s, 2H), 6.82 (d, *J* = 8.9 Hz, 2H), 5.24 (s, 2H), 4.84 (s, 2H), 4.34 (ddd, *J* = 9.7, 7.8, 5.0 Hz, 1H), 3.19 (s, 3H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.11 – 1.99 (m, 1H), 1.99 – 1.79 (m, 1H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 173.9, 173.7, 166.3, 162.0, 157.8, 155.1, 151.0, 150.7, 150.3, 148.0, 137.7, 134.2, 129.0, 127.0, 121.3, 120.9, 111.1, 66.7, 54.9, 51.7, 39.0, 30.4, 26.0; HRMS (ESI) *m/z*: calcd for $C_{28}H_{28}BN_8O_9$ $[M-H]^-$ 631.2078, found 631.2028.

**4-(((Benzyloxy)carbonyl)amino)benzoic acid (19).** To a solution of 4-methylaminobenzoic acid **18** (4.11 g, 29.9 mmol) and $NaHCO_3$ (22.6 g, 269 mmol) in a mixture of $H_2O$/THF (100 mL, 1/1, v/v) was added benzyl chloroformate (4.26 mL, 29.9 mmol) dropwise at 0 °C. The reaction mixture was stirred for 4 h at 21 °C followed by addition of 20 mL of water. Then, the mixture was stirred for an additional 16 h followed by acidification with 1 M aq. HCl to pH ~ 3. Next, the precipitate was filtered off and dried *in vacuo* to afford **19** as a white solid (7.2 g, 89%). m.p.: 213.0–217.0 °C (lit.[72] 217 °C, decomp.); IR (neat, $cm^{-1}$): 3324.4, 2951.7, 2677.4, 2557.2, 1702.1, 1672.4, 1528.6, 1512.8, 1225.8, 1062.6; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.66 (br s, 1H), 10.15 (s, 1H), 7.87 (d, *J* = 8.7 Hz, 2H), 7.58 (d, *J* = 8.7 Hz, 2H), 7.51 – 7.17 (m, 5H), 5.17 (s, 2H);[73] $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 167.0, 153.2, 143.3, 136.3, 130.5, 128.5, 128.2, 128.2, 124.5, 117.3, 66.1.[74]

**Dimethyl (4-(((benzyloxy)carbonyl)amino)benzoyl)-L-glutamate (20).** To a suspension of **19** (2.90 g, 10.7 mmol) in anhydrous $CH_2Cl_2$ was added 1 drop of DMF followed by thionyl chloride (3.88 mL, 53.4 mmol) and the reaction mixture was refluxed for 24 h under an $N_2$

atmosphere. Next, all volatiles were removed *in vacuo* to afford the acyl chloride as a pale yellow solid (3.1 g, >95%) which was added to a stirred mixture of **9**·HCl (2.40 g, 11.3 mmol) and $Et_3N$ in anhydrous $CH_2Cl_2$ (100 mL) and an additional 2 h stirring was applied under an $N_2$ atmosphere at 21 °C. The mixture was then diluted with EtOAc (250 mL), washed with 1 M aq. HCl (2 x 150 mL), sat. aq. $NaHCO_3$ (2 x 150 mL), and brine (200 mL), dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford the title compound **20** as a white solid (4.3 g, 97%). m.p.: 121.7–124.0 °C; $[\alpha]_D^{20}$ = -16.2 (c 1.0, MeOH); IR (neat, $cm^{-1}$): 3378.3, 3297.8, 2952.4, 1730.5, 1627.7, 1526.8, 1227.4; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.07 (s, 1H), 8.60 (d, *J* = 7.4 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 2H), 7.55 (d, *J* = 8.8 Hz, 2H), 7.50 – 7.26 (m, 5H), 5.18 (s, 2H), 4.44 (ddd, *J* = 9.5, 7.4, 5.4 Hz, 1H), 3.64 (s, 3H), 3.59 (s, 3H), 2.45 (t, *J* = 7.5 Hz, 2H), 2.22 – 1.83 (m, 2H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.7, 172.4, 166.1, 153.2, 142.1, 136.4, 128.5 (2C), 128.2, 128.1, 127.3, 117.2, 66.0, 51.9, 51.9, 51.4, 29.9, 25.7; HRMS (ESI) *m/z*: calcd for $C_{22}H_{25}N_2O_7$ $[M+H]^+$ 429.1656, found 429.1676.

**Dimethyl (4-aminobenzoyl)-L-glutamate hydrochloride (21**·HCl**).** A solution of **20** (2.0 g, 4.8 mmol) in anhydrous MeOH (100 mL) was treated with Pd/C (200 mg, 10% w/w). The mixture was stirred for 12 h under a $H_2$ atmosphere at 21 °C, then filtered through a path of Celite®, concentrated *in vacuo* over silica gel and purified by flash column chromatography on silica gel using a mixture of EtOAc/Hep (2/1, v/v) as the eluent to give a clear oil corresponding to the free amine **21**. Treatment with 2 M HCl in ether afforded the hydrochloride salt **21**·HCl as a white solid (1.3 g, 95%). $R_f$ = 0.38 (free amine; EtOAc/Hep, 4/1, v/v); m.p.: 65.2 – 70.0 °C; $[\alpha]_D^{20}$ = -17.8 (c 1.26, $H_2O$); IR (neat, $cm^{-1}$): 3270.9, 2952.3, 2834.2, 2577.2, 1731.4, 1641.3, 1609.8, 1538.9, 1501.4, 1436.4, 1206.7, 1171.7; $^1$H NMR (400 MHz, $D_2O$) δ 7.78 (d, J = 8.5 Hz, 2H), 7.33 (d, J = 8.5 Hz, 2H), 4.58 (dd, J = 9.4, 5.2 Hz, 1H), 3.70 (s, 3H), 3.56 (s, 3H), 2.49 (t,

J = 7.0 Hz, 2H), 2.35 – 2.20 (m, 1H), 2.10 (ddt, J = 14.2, 9.4, 6.9 Hz, 1H); $^{13}$C NMR (101 MHz, $D_2O$) δ 175.8, 173.7, 169.8, 136.8, 131.5, 129.2, 121.9, 53.0, 52.7, 52.3, 30.2, 25.5; HRMS (ESI) m/z: calcd for $C_{14}H_{19}N_2O_5$ $[M+H]^+$ 295.1288, found 295.1297.

**(*S*)-(4-(((4-((1,5-Dimethoxy-1,5-dioxopentan-2-yl)carbamoyl)phenyl)amino)methyl)phenyl) boronic acid (22).** To a solution of **21**·HCl (0.80 g, 2.42 mmol) in anhydrous MeOH was added (4-formylphenyl)boronic acid (0.55 g, 3.67 mmol) followed by portion-wise addition of $NaBH_3CN$ (0.80 g, 12.1 mmol). The reaction mixture was stirred for 12 h under an $N_2$ atmosphere at 21 $^oC$. Extra (4-formylphenyl)boronic acid (0.55 g, 3.67 mmol) was then added to the stirred mixture followed by an additional 24 h of stirring for completion of the reaction. Next, the crude mixture was concentrated *in vacuo*, redissolved in EtOAc (150mL), and washed with sat. aq. $NaHCO_3$ (2 x 150 mL) and brine (150 mL). The organic phase was dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* to afford a pale yellow solid. Purification by reversed-phase preparative HPLC afforded the title compound **22** as a white solid (699 mg, 67%). $R_f$ = 0.77 (silica, MeOH/$CH_2Cl_2$, 1/1, v/v); m.p.: 84.1–88.2 $^oC$; $[\alpha]_D^{20}$ = -16.3 (c 1.04, DMSO); IR (neat, $cm^{-1}$): 3382.1, 2951.9, 1726.9, 1601.9, 1506.4, 1330.4; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.24 (d, J = 7.5 Hz, 1H), 7.98 (s, 2H), 7.73 (d, J = 7.8 Hz, 2H), 7.62 (d, J = 8.6 Hz, 2H), 7.30 (d, J = 7.8 Hz, 2H), 6.84 (t, J = 6.0 Hz, 1H), 6.58 (d, J = 8.6 Hz, 2H), 4.38 (ddd, J = 9.5, 7.5, 5.6 Hz, 1H), 4.33 (d, J = 6.0 Hz, 2H), 3.61 (s, 3H), 3.57 (s, 3H), 2.41 (t, J = 7.4 Hz, 2H), 2.14 – 2.03 (m, 1H), 2.02 – 1.86 (m, 1H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.8, 172.7, 166.6, 151.4, 141.6, 134.2, 129.0, 126.2, 120.4, 111.1, 51.8, 51.7, 51.4, 46.0, 30.0, 25.8; HRMS (ESI) m/z: calcd for $C_{21}H_{26}BN_2O_7$ $[M+H]^+$ 429.1828, found 429.1850.

**(*S*)-(4-((((2,4-Diaminopteridin-6-yl)methyl)(4-((1,5-dimethoxy-1,5-dioxopentan-2-yl)carbamoyl)phenyl)amino)methyl)phenyl)boronic acid (24).** A solution of **22** (461 mg, 1.08

mmol) and 6-(bromomethyl)pteridine-2,4-diamine hydrobromide **23**·HBr (515 mg, 1.38 mmol) in anhydrous DMA (4.85 mL) was stirred for three days under an $N_2$ atmosphere at 55 °C. Then, $Et_3N$ (79 µL, 3.21 mmol) was added to the mixture followed by addition of $H_2O$ (approx. 30 mL). The orange precipitate was filtered off and purified by reversed-phase preparative HPLC to afford **24** as a yellow solid (279 mg, 43%). $R_f$ = 0.47 (silica, $CH_2Cl_2$/MeOH, 85/15, v/v); m.p.: >180 °C (decomp.); $[\alpha]_D^{20}$ = +1.75 (c 0.57, DMSO-$d_6$); IR (neat, $cm^{-1}$): 3485.2, 3327.3, 3169.7, 2952.1, 1727.2, 1625.3, 1602.2, 1201.7; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.64 (s, 1H), 8.31 (d, *J* = 7.5 Hz, 1H), 7.99 (s, 2H), 7.75 (d, *J* = 8.0 Hz, 2H), 7.72 – 7.60 (m, 3H), 7.47 (br s, 1H), 7.23 (d, *J* = 8.0 Hz, 2H), 6.82 (d, *J* = 9.1 Hz, 2H), 6.63 (br s, 2H), 4.93 (s, 2H), 4.89 (s, 2H), 4.38 (ddd, *J* = 9.6, 7.5, 5.3 Hz, 1H), 3.61 (s, 3H), 3.57 (s, 3H), 2.41 (t, *J* = 7.5 Hz, 2H), 2.17 – 1.79 (m, 2H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.72, 172.54, 166.38, 162.83, 162.71, 155.18, 150.37, 149.36, 145.92, 140.38, 134.39, 128.93, 125.66, 121.41, 121.10, 111.48, 54.47, 53.98, 51.82, 51.71, 51.35, 29.92, 25.77; HRMS (ESI) *m/z*: calcd for $C_{28}H_{32}BN_8O_7$ $[M+H]^+$ 603.2482, found 603.2508.

**(4-((4-Boronobenzyl)((2,4-diaminopteridin-6-yl)methyl)amino)benzoyl)-L-glutamic acid (25).** A solution of **24** (46 mg, 76 µmol) in 1 M aq. NaOH (2mL) was stirred for 5 min at 21 °C. The crude was directly purified by reversed-phase preparative HPLC to afford the title compound **25** as a yellow solid (17 mg, 40%). m.p.: >245 °C (decomp.); $[\alpha]_D^{20}$ = +7.8 (c 0.63, DMSO); IR (neat, $cm^{-1}$): 3336.5, 3089.8, 1633.5, 602.7, 1502.8, 1394.2, 1364.9, 1337.0, 1202.5; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.63 (s, 1H), 8.08 (d, *J* = 7.3 Hz, 1H), 8.01 (s, 2H), 7.73 (d, *J* = 7.9 Hz, 2H), 7.69 – 7.55 (m, 3H), 7.46 (br s, 1H), 7.22 (d, *J* = 7.9 Hz, 2H), 6.81 (d, *J* = 8.9 Hz, 2H), 6.61 (br s, 2H), 4.91 (s, 2H), 4.88 (s, 2H), 4.32 (ddd, *J* = 9.7, 7.9, 5.1 Hz, 1H), 2.3 (t, *J* = 7.5, 2H), 2.02 – 1.78 (m, 2H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 174.0, 173.7, 166.2, 162.9,

162.7, 155.2, 150.3, 149.4, 145.9, 140.4, 134.4, 128.8, 125.7, 121.5, 121.4, 111.5, 54.4, 54.0, 51.8, 30.6, 26.2; HRMS (ESI) *m/z*: calcd for $C_{26}H_{28}BN_8O_7$ $[M+H]^+$ 575.2169, found 575.2187.

**Physicochemical and pharmacokinetic *in vitro* assays**

*Activation of prodrugs under oxidative conditions.* To a mixture of DMSO (150 µL) and aq. PBS buffer (650 µL, pH 7.4) in a microcentrifuge tube (1.5 mL) placed in a thermomixer at 37 $^oC$ was added a solution of prodrug (50 µL, 1 mM in DMSO) followed by addition of internal standard solution (diclofenac, 50 µL, 1 mM in DMSO). The assay was initiated by addition of a solution of $H_2O_2$ in PBS (50 µL, 10 mM) followed by vortex mixing. The resulting mixture was incubated at 37 $^oC$ in an Eppendorf Thermomixer C (1.5 mL, 1000 rpm) and samples were taken after 5, 15, 30, 60, 90 min and 2, 3, and 4 h. Analysis of the percentage of remaining compound was performed using RP-UPLC-UV (λ= 306 nm). Further data points were collected for compounds **24** and **25**, including 16, 24 and 48 h. A control experiment (no $H_2O_2$ addition but PBS) was performed in parallel. Every prodrug activation assay was carried out in triplicates.

*Activation of prodrugs under different $H_2O_2$ concentrations.* To aq. PBS buffer (750 µL, pH 7.4) was added a solution of prodrug **17** or **25** (100 µL, 1 mM in DMSO) followed by addition of internal standard solution (diclofenac, 100 µL, 1 mM in DMSO). The assay was initiated by addition of a solution of $H_2O_2$ in PBS (50 µL, 10 - 5 – 1 – 0.5 – 0.25 mM) followed by vortex mixing. The resulting mixture was incubated at 37 $^oC$ in a Eppendorf Thermomixer C (1.5 mL, 1000 rpm) and samples were taken after 5, 15, 30, 60, 90 min and 2, 4, 24 h (48 h for compound **25**). Analysis of the percentage of remaining compound was performed using RP-UPLC-UV (λ= 306 nm). A control experiment (no $H_2O_2$ addition but PBS) was performed in parallel. Every prodrug activation assay was carried out in triplicates.

*Kinetic solubility.* The kinetic solubility of prodrugs **11**, **12**, **13**, **17**, **24**, and **25** was determined in a 1% DMSO in PBS solution at a concentration of 100 μM. A solution of test compound (10 μL, 10 mM) was added to an microcentrifuge tube containing PBS (990 μL, pH 7.4), and the mixture was incubated at 37 °C for 20 hours in a Eppendorf Thermomixer C (1.5 mL, 1000 rpm). After 20 h incubation, the samples were centrifuged at 5000 rpm (37 °C, 30 min) to pellet insoluble material. Aliquots (250 μL) of the supernatant were diluted in PBS (250 μL) and the concentration of dissolved compound was quantified by RP-UPLC-UV (λ= 306 nm) analysis. Solubility determinations were performed in triplicates.

*Chemical Stability pH 7.4.* The chemical stability at pH 7.4 of compounds **17** and **25** was assessed using diclofenac as an internal standard. To 490 μL of a pre-warmed solution of 20 μM diclofenac in PBS (0.1% DMSO) was added 10 μL of a 1 mM DMSO solution of compound **17** and **25** (in triplicates) and RP-UPLC-UV (λ= 306 nm) analysis was performed after 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, and 48 h.

*Simulated gastric and intestinal fluid stability.* Simulated gastric fluid (SGF) was generated by preparing a solution of sodium chloride (0.20 g), concentrated HCl (0.70 mL), and pepsin (Sigma, P-7000, 0.32 g) in deionized water (95 mL) up to a 100 mL of final volume. Simulated instestinal fluid (SIF) was generated by preparing a solution of monobasic potassium phosphate ($KH_2PO_4$, 0.68 g), 0.1 N aqueous sodium hydroxide (38 mL), and pancreatin (Sigma, P-1625, 1.0 g) in deionized water up to 100 mL of final volume. The pH of the solution was adjusted to 7.5 by addition of 1 N aqueous sodium hydroxide.[75] To 990 μL of SGF or SIF was added a solution of prodrug **17** or **25** (10 μL, 10 mM in DMSO). The resulting mixture was incubated at 37 $^{o}$C in a Eppendorf Thermomixer C (1.5 mL, 1000 rpm) and samples were taken (90 μL) after 0, 1, 2, 4, and 24 h. Diclofenac (10 μL, 1 mM in DMSO) was added to the taken samples as internal

standard. Analysis of the percentage of remaining compound was performed using RP-UPLC-UV (λ= 306 nm). A negative control experiment (no pepsin or pancreatin addition) was performed in parallel. Every assay was carried out in triplicates.

*Human and mouse plasma stability.* 2 mL of lyophilized plasma (Sigma, P9523 for human and P9275 for mouse plasma) were dissolved in 2 mL of milli-Q water. To an microcentrifuge tube containing 240 μL of pre-warmed PBS (37 $^{o}$C) was added 250 μL of plasma. The mixture was shaken at 300 rpm in an Eppendorf Thermomixer C (1.5 mL, 300 rpm) for 10 min before the assay was started. A solution of test compound **17** and **25** in DMSO (1 mM, 10 μL) was added to the pre-warmed PBS/plasma mixture to a final concentration of 20 μM. Samples (50 μL) were taken after 30 min, 1h, 2h, 4h, 8h, 24h and 48h and added to 150 μL of ice-cold MeOH containing an internal standard (diclofenac, 10 μM). Procaine and dilizatem were used as positive controls for human and mouse plasma stability, respectively. The half-lives ($t_{1/2}$) was determined using GraphPad Prism 6 software and calculated with the slope of the linear region of the plotted logarithm of remaining compound against time, assuming first order kinetics.

*Human and mice microsomal stability.* The *in vitro* microsomal metabolism of compounds **17** and **25** was evaluated in isolated liver microsomes (pooled, from CD-1 male mouse and from human male) at a concentration of 10 μM. A solution of liver microsomes (10 mg/mL, 25 μL) was added to an microcentrifuge tube containing 425 μL of PBS at 37 $^{o}$C in an Eppendorf Thermomixer C (1.5 mL, 1000 rpm) and the mixture was shaken for 10 min before the actual assay was started. Then, a DMSO solution of test compound (5 μL, 1 mM) was added followed by an aq. solution of NADPH (25 μL) for initiation of the experiment. A negative (no NADPH) and a positive control experiments (verapamil and diphenhydramine for human and mouse, respectively) were carried out in parallel. Aliquots (50 μL) were removed after 0 min, 5 min, 10

min, 15 min, 30 min, 45 min, and 60 min (additional 120 min and 240 min time points for the mice study) and quenched immediately with 50 µL of ice-cold MeCN containing an internal standard (diclofenac 10 µM). Samples were analysed by RP-UPLC-UV and half-lives calculated plotting the Ln [C] versus time, assuming first order kinetics using GraphPad Prism 6.

**Cellular Assays**

*Materials.* Dulbecco's Phosphate buffer (D-PBS) was purchased from Sigma Aldrich (D8662, pH 7.4). DMEM (D5976, Sigma Aldrich) and RPMI (R8758, Sigma Aldrich) were supplemented with 10% foetal bovine serum (FBS, heat-inactivated, Fisher Scientific), penicillin at 100 units/mL, and streptomycin at 100 mg/mL (all purchased from Sigma Aldrich). The CellTiter 96 ® AQueous Non-Radioactive Cell Proliferation Assay was purchased from Promega (MTS, G5421).

*Cell Culture.* The human breast cancer MCF-7 (Sigma) and human large cell lung cancer NCIH-460 (ATTC) cell lines were cultured in a humidified, 5% $CO_2$ atmosphere at 37 $^{o}$C in Dulbecco´s Modified Eagle´s Medium (DMEM) (Sigma) or Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma) supplemented with 10% foetal bovine serum (FBS, heat-inactivated, Fisher Scientific) and 1% penicillin/streptomycin. Both cell lines were subcultured every 2-3 days.

*Pre-activation of compounds with $H_2O_2$.* A 125 µM solution of tested compound was prepared in a 1.25 mM $H_2O_2$ in DMSO:PBS (1:1), placed in an microcentrifuge tube and shaken at 21 $^{o}$C for 24 h at 1000 rpm in an Eppendorf Thermomixer C (1.5 mL). The activation was followed by RP-UPLC-MS (λ = 306 nm) after 0 min, 15 min, 1 h, 4 h, and 24 h. A negative control

consisting of a 125 μM solution of compound in a mixture of DMSO:PBS (1:1) without $H_2O_2$ was run in parallel to the activation assay under the same conditions.

*Evaluation of cell viability.* The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-te-trazolium (MTS) assay (Promega Biotech AB, Stockholm, Sweden ) was used to determine the in vitro antiproliferative effect of the compounds. This assay is based on the principle that cells have the ability to reduce MTS tetrazolium, while, when dead, they lose this ability. MCF-7 and NCI-H460 cells were cultured in 96-well plates at an initial density of $10^4$ cells/well (MCF-7) or $7x10^3$ cells/well (NCI-H460) in their respective growth medium. After 24 h incubation to allow cell attachment, the medium was removed and the cells were incubated in the presence or absence of pre-activated compounds at different concentrations. After 48 h incubation time, the MTS reagent was added to each well. The cells were further incubated for a period of time between 30-60 min at 37 ºC until colorimetric reaction was developed within the linear range and the absorbance of the samples was measured at 490 nm using a 96-well plate spectrophotometer (Victor 3 plate reader with Wallac 1420 Workstation vs 3.0 software).

A control was used for each tested compound, where cells were incubated with DMEM or RPMI containing the equivalent concentration of DMSO (maximum of 0.4% v/v). Each concentration of tested compounds was done in triplicates. The final concentration of $H_2O_2$ in each well was always <10 μM (non-cytotoxic concentration in MCF-7 and NCI-H460 cell lines, determined with the described assay). The $IC_{50}$ values were calculated using GraphPad Prism v6.0 (California, USA) as the concentration of the compounds required to cause 50% response compared to cells exposed to controls using a non-linear dose-response regression.

**Collagen type-II-induced arthritis *in vivo* efficacy and preliminary toxicity assay**

*Animals*: DBA/1J mice (male, 8–9 weeks) were obtained from Janvier, France. The mice were maintained in the animal facility at Redoxis, Medicon Village, Lund, Sweden, where they were acclimatized for approximately one week before initiation of the experiment. All animal experiments were approved by the local animal ethic committee Malmö/Lund, Sweden, under the license N165-15.

*Induction of disease: collagen induced arthritis* (CIA) was induced by intradermal immunization with 100 µg of chicken type-II collagen (CII, Chondrex) in Complete Freund's Adjuvant (CFA, Difco) on day -1 *via* subcutaneous injection approximately 0.5 cm from the base of the tail. On day 21 a boost injection was administered in the same way with 50 µg CII. One week after the second immunization, onset of disease started to be observed (day 26).

*Anti-arthritic effect of test compounds and health evaluation*: mice were randomly divided in 5 groups (n = 8 per group): group I (vehicle), group II (**MTX**, Sigma Aldrich, 7.0 mg/kg, *i.p.*), group III (**AMT**, Enzo Life Sciences, 6.8 mg/kg, *i.p.*), group IV (**17**, 9.7 mg/kg, *i.p.*), group V (**25**, 8.8 mg/kg, *i.p.*). Vehicle or test compound (2% DMSO in PBS, Life Technologies, injection volume 370 µL) were dosed daily by intraperitoneal injections for 14 days, starting at onset of disease (day 27). Disease was evaluated three times per week in a blinded fashion, starting at day 18 until the end of the experiment (day 40). A macroscopic scoring system of the four limbs ranging from 0 to 15 (1 point per swollen toe, 1 point per swollen foot knuckle, and 5 points for swollen ankle) was used, meaning a maximal score of 60 per mice. For humane and ethical reasons and restrictions, mice with scores exceeding 45 were euthanized. The general health of mice was evaluated three times per week after disease induction. As an indicator of general health, animal body weight was used.

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**Author Contributions**

JPC and VP performed experiments. JPC, JB, CAH, VP, AEH, TLA and MHC designed the study, analyzed data and wrote the manuscript. All authors have approved the final version of the manuscript.

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ABBREVIATIONS

AMT, aminopterin; ADMET, absorption, distribution, metabolism, excretion, and toxicity; CIA, collagen-induced arthritis; $CL_{int}$, intrinsic clearance; DMARD; disease modifying anti-rheumatic drug; LD-MTX, low-dose methotrexate; ROS, reactive oxygen species; RP-UPLC-MS, reversed-phase ultra-performance liquid chromatography; MTX, methotrexate; PBS, phosphate buffered saline; RA, rheumatoid arthritis; SEM, standard error mean; TLC, thin layer chromatography.

ASSOCIATED CONTENT

**Supporting Information**. Hydrogen peroxide prodrug activation, cell viability, chemical, SGF, SIF, plasma, and metabolic stabilities assays and assigned NMR spectra of synthesized

compounds, as well as a molecular formula strings file. This material is available free of charge via the Internet at http://pubs.acs.org.

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