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Synthesis of β-Hydroxy-Propenamide Derivatives and the Inhibition of Human Dihydroorotate Dehydrogenase

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Novel β-hydroxy propenamides as analogues of the active metabolite of leflunomide (A 771726) were synthesized and evaluated for their inhibitory activity on dihydroorotate dehydrogenase (DHODH) in an investigation into their immunosuppressive activity. Compounds **2a**, **3a**, and **3h** were approximately 4-40 times more potent than leflunomide in their activity while they were less active than A 771726.

Key words: Immunosuppressive activity, A 771726, Leflunomide, Succinyl acetone, β-Hydroxy propenamides, Dihydroorotate dehydrogenase (DHODH)

INTRODUCTION

Immunosuppressive drugs such as azathioprine (Elison, *et al.*, 1975), cyclosporin A (CsA) (Borel, *et al.*, 1982), and FK-506 (Tanaka, *et al.*, 1987) are used widely in the area of possible organ transplantation and the treatment of autoimmune diseases. However, all these agents, apart from a significant proportion of failures, show a number of undesired side effects including nephrotoxicity, hypertention, neurological disorders, gingival overgrowth, hirsutism, *etc* (Shaw, *et al.*, 1996; Perico, *et al.*, 1997). Consequently, there is a need for newer less toxic compounds and with new mechanisms of action, which could lead to a more specific immunosuppression regime.

Dihydroorotate dehydrogenase (DHODH) catalyzes the fourth step in pyrimidine biosynthesis, which is the conversion of dihydroorotate to orotic acid (Jones, *et al.*, 1980). Its inhibition would lead to a pyrimidine nucleotide depletion and the resulting inhibition of DNA and RNA synthesis and cell proliferation. Cell proliferation is a vital component of the immune response, during which the immune cells have a high nucleotide requirement. Therefore, a DHODH inhibitor would be a potential immunosuppressive agent. Leflunomide, an immunosuppressant, is a new disease-modifying anti-rheumatic drug approved for treating rheumatoid arthritis (RA) (Sanders, *et al.*, 2002), which acts *via* DHODH inhibition. The isoxazole immunosuppressants leflunomide is quickly metabolized into its ring-opened isomer, A 771726 (Fig. 1), which inhibits the enzymatic activity of DHODH as an active metabolite (Davis, *et al.*, 1996). Therefore, metabolite A 771726 is a potential candidate for the clinical development in areas where it is inappropriate to develop leflunomide itself. Westwood *et al.* reported the synthesis and structure activity relationship of a series of molecules related to the active metabolite in order to identify analogues with an equivalent activity to leflunomide (Kuo, *et al.*, 1996). This metabolite has a minimum structural and physicochemical features required for the efficient inhibition of human DHODH by the α-cyano-β-hydroxy propenamide building blocks. In addition, it has a practically planar structure with the amide carbonyl and the enolic hydroxyl forming a strong hydrogen bond (Papageorgiou, *et al.*, 1997; Bertolini, *et al.*, 1997). Therefore, the activity in the enamide series is critically dependent on the role of the enolic hydroxyl group.

Succinylacetone (SA) is a seven carbon organic ketoacid. It is a new immunosuppressive compound, which was first noted in the urine of patients with hereditary tyrosinemia due to a fumarylacetoacetate hydrolase deficiency (Lindblad, *et al.*, 1977). The immunosuppressive effects of SA in terms of its ability to inhibit tumor allograft rejection (Tschudy, *et al.*, 1981), marked immunosuppression in acute graft-*vs.*-host disease (GVHD) (Hess, *et al.*, 1987; Fidler, *et al.*,

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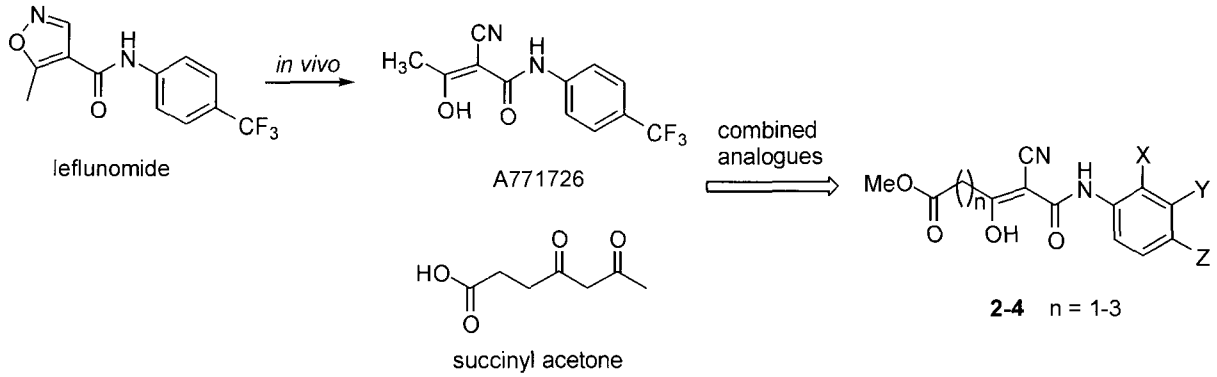


Fig. 1. Structure of leflunomide, A 771726, succinyl acetone, and the designed compounds.

1993), and experimental autoimmune uveitis (Skolik, *et al.*, 1988) were discovered. The SA structure has an enolic hydroxyl group similar to the metabolite of leflunomide. With this in mind, this study designed novel structure **2-4**, α-cyano-β-hydroxy propenamide, as structurally combined analogues of the corresponding possible structures of leflunomides bioactive metabolites and immunosuppressant succinyl acetone. The analogues **2-4** can be classified into two subgroups; 3-substituted carboxyalkyl groups as a side chain and substituted aromatic groups.

MATERIALS AND METHODS

Unless otherwise noted, all the materials were obtained from commercial suppliers and were used without purification. All the reactions requiring anhydrous conditions were performed in oven-dried glassware under a N_2 atmosphere. Tetrahydrofuran (THF) was distilled from sodium-benzophenone immediately prior to use. Thin layer chromatography (TLC) was carried out using Merck Silica Gel 60 precoated plates. The melting points were measured using a capillary melting point apparatus (Thomas Scientific Co.) and were not corrected. ^{1}H-NMR was recorded on a Bruker AMX 300 and is reported in ppm downfield from tetramethylsilane (δ 0.00). The IR spectra were recorded on a Jasco 430 infrared spectrophotometer and reported as cm^{-1}. The recombinant truncated human DHODH (43 kDa; EMBL sequence data bank Accession No. M94065) was purified as previously described (Knecht, *et al.*, 1996).

General procedure for the synthesis of the β-hydroxy enamides 2-4

A solution of the cyanoacetamide (2.0 mmol) (Papageorgiou, *et al.*, 1997) and sodium hydride (8.4 mmol) in dry THF (10 mL) was stirred for 10 min in an ice bath and for 30 min at room temperature. Subsequently, acyl chloride (2.2 mmol) was added to the reaction mixture. After 60 min, the reaction mixture was quenched with acetic acid (0.4 mL) and 0.3 N HCl (16 mL). The precipitated solid was filtered and washed with ether to give a white solid.

Methyl 4-cyano-3-hydroxy-5-oxo-5-[[4-(trifluoromethyl) phenyl]amino]-3-pentenoate (2a)

Yield 42%; mp : 193-194; R_f = 0.2-0.3 (ethyl acetate); ^{1}H-NMR ($CDCl_3$) 15.74 (1H, s, OH), 7.72(1H, bs, NH), 7.65 (4H, bs, phenyl), 3.80 (3H, s, CH_3O), 3.68 (2H, s, $COCH_2$); IR (KBr) 3400 (OH, w), 2360 (CN, s), 1735 (C=O, s), 1604 (C=C, m), 1112 (COC, s).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[[4-(trifluoromethyl) phenyl]amino]-4-hexenoate (3a)

Yield 53%; mp : 172-173; ^{1}H-NMR ($CDCl_3$) 15.74 (1H, s, OH), 7.72 (1H, bs, NH), 7.64 (4H, bs, phenyl), 3.73 (3H, s, CH_3O), 2.97-3.01 (2H, m, $CO\underline{CH_2}CH_2$), 2.74-2.79 (2H, m, $COCH_2\underline{CH_2}$); IR (KBr) 3295 (OH, w), 2221(CN, s), 1735(C=O, s), 1635(C=C, m), 1112(COC, s).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[[3-(trifluoromethyl) phenyl]amino]-4-hexenoate (3b)

Yield 51%; mp 128-130; ^{1}H-NMR ($CDCl_3$) 15.84 (1H, s, OH), 7.89 (1H, s, NH), 7.26-7.89 (4H, m, phenyl), 3.72 (3H, s, CH_3O), 2.96-3.01 (2H, m, $CO\underline{CH_2}CH_2$), 2.74-2.78 (2H, m, $COCH_2\underline{CH_2}$); IR (KBr) 2217 (CN, s), 1747 (C=O, s), 1590 (C=C, m), 1126 (COC, s).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[[2-(trifluoromethyl) phenyl]amino]-4-hexenoate (3c)

Yield 50%; ^{1}H-NMR ($CDCl_3$) 15.58 (1H, s, OH), 7.98 (1H, s, NH), 7.36-7.95 (4H, m, phenyl), 3.69 (3H, s, CH_3O), 2.97-3.02 (2H, m, $CO\underline{CH_2}CH_2$), 2.73-2.78 (2H, m, $COCH_2\underline{CH_2}$); IR (KBr) 3419 (NH, w), 3295 (OH, w), 2210 (CN, s), 1741(C=O, s), 1587(C=C, m), 1114 (COC, s).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[4-(chlorophenyl) amino]-4-hexenoate (3d)

Yield 57%; mp : 155; ^{1}H-NMR ($CDCl_3$) 15.95 (1H, s, OH), 7.73 (1H, s, NH), 7.35-7.43 (4H, m, phenyl), 3.73

(3H, s, CH_3O), 2.97 (2H, bs, $CO\underline{CH_2}CH_2$), 2.75 (2H, bs, $COCH_2\underline{CH_2}$); IR (KBr) 3291 (OH, w), 2217 (CN, m), 1729 (C=O, s) 1600 (C=C, s), 1390 (COC, m).

Methy 5-cyano-4-hydroxy-6-oxo-6-[3-(chlorophenyl) amino]-4-hexenoate (3e)

Yield 56%; mp : 112-113; ^{1}H-NMR ($CDCl_3$) 15.82 (1H, s, OH), 7.64 (1H, s, NH), 7.16-7.64 (4H, m, phenyl), 3.73 (3H, s, CH_3O), 2.96-3.00 (2H, m, $CO\underline{CH_2}CH_2$), 2.74-2.78 (2H, m, $COCH_2\underline{CH_2}$); IR (KBr) 3295 (OH, w), 2221 (CN, s), 1735 (C=O, s), 1583 (C=C, m), 1191 (COC, w).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[4-(methylphenyl) amino]-4-hexenoate (3f)

Yield 57%; mp : 132-133; ^{1}H-NMR ($CDCl_3$) 16.17 (1H, s, OH), 7.59 (1H, s, NH), 7.16-7.35 (4H, m, phenyl), 3.72 (3H, s, CH_3O), 2.94-2.99 (2H, m, $CO\underline{CH_2}CH_2$), 2.72-2.76 (2H, m, $COCH_2\underline{CH_2}$), 2.34 (3H, s, CH_3); IR (KBr) 3286 (OH, w), 2225 (CN, s), 1735 (C=O, s), 1600 (C=C, s), 1168 (COC, w).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[4-(cyanophenyl) amino]-4-hexenoate (3g)

Yield 52%; mp : 175-176; ^{1}H-NMR ($CDCl_3$) 15.57 (1H, s, OH), 7.73 (1H, s, NH), 7.63-7.70 (4H, m, phenyl), 3.73 (3H, s, CH_3O), 2.97-3.02 (2H, m, $CO\underline{CH_2}CH_2$), 2.74-2.79 (2H, m, $COCH_2\underline{CH_2}$); IR (KBr) 3282 (OH, w), 2225 (CN, s), 1729 (C=O, s), 1639 (C=C, s), 1199 (COC, w).

Methyl 5-cyano-4-hydroxy-6-oxo-6-[4-(methylsulfenyl-phenyl)amino]-4-hexenoate (3h)

Yield 53%; mp : 136-137; ^{1}H-NMR ($CDCl_3$) 16.01 (1H, s, OH), 7.41 (1H, s, NH), 7.24-7.38 (4H, m, phenyl), 3.72 (3H, s, CH_3O), 2.95-2.99 (2H, m, $CO\underline{CH_2}CH_2$), 2.72-2.77 (2H, m, $COCH_2\underline{CH_2}$), 2.49 (3H, s, SCH_3); IR (KBr) 3455 (NH, w), 3295 (OH, w), 2213 (CN, s), 1739 (C=O, s), 1573 (C=C, s), 1126 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[[4-(trifluoromethyl) phenyl]amino]-5-heptenoate (4a)

Yield 56%; mp : 162-163; ^{1}H-NMR ($CDCl_3$) 15.57 (1H, s, OH), 7.85 (1H, s, NH), 7.65 (4H, bs, phenyl), 3.71 (3H, s, CH_3O , 2.68-2.73 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.42-2.47 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.05-2.10 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) : 3291 (OH, w), 2213 (CN, s), 1725 (C=O, s), 1552 (C=C, s), 1122 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[[3-(trifluoromethyl) phenyl]amino]-5-heptenoate (4b)

Yield 53%; mp : 133-134; ^{1}H-NMR ($CDCl_3$) 15.53 (1H, s, OH), 7.86 (1H, s, NH), 7.48-7.51 (4H, m, phenyl), 3.71 (3H, s, CH_3O), 2.68-2.73 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.42-2.47 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.05-2.10 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3295 (OH, w), 2225 (CN, s), 1739 (C=O, s), 1600 (C=C, s), 1112 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[[2-(trifluoromethyl) phenyl]amino]-5-heptenoate (4c)

Yield 51%; ^{1}H-NMR ($CDCl_3$) 15.41 (1H, s, OH), 8.03 (1H, s, NH), 7.36-7.95 (4H, m, phenyl), 3.68 (3H, s, CH_3O), 2.67-2.72 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.41-2.47 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.03-2.08 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3423 (NH, w), 3286 (OH, w), 2213 (CN, s), 1737 (C=O, s), 1590 (C=C, s), 1124 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[4-(chlorophenyl) amino]-5-heptenoate (4d)

Yield 56%; mp : 134-135; ^{1}H-NMR ($CDCl_3$) 15.74 (1H, s, OH), 7.82 (1H, s, NH), 7.32-7.47 (4H, m, phenyl), 3.69 (3H, s, CH_3O), 2.66-2.71 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.41-2.46 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.03-2.08 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3309 (NH, w), 3268 (OH, w), 2217 (CN, s), 1735 (C=O, s), 1629 (C=C, s), 1178 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[3-(chlorophenyl) amino]-5-heptenoate (4e)

Yield 58%; mp : 120-121; ^{1}H-NMR ($CDCl_3$) 15.56 (1H, s, OH), 7.66 (1H, s, NH), 7.16-7.65 (4H, m, phenyl), 3.70 (3H, s, CH_3O), 2.67-2.72 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.42-2.47 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.04-2.09 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3417 (NH, w), 3295 (OH, w), 2221 (CN, s), 1735 (C=O, s), 1587 (C=C, s).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[4-(methylphenyl) amino]-5-heptenoate (4f)

Yield 30%; mp : 95-96; ^{1}H-NMR ($CDCl_3$) 15.86 (1H, s, OH), 7.51 (1H, s, NH), 7.16-7.36 (4H, m, phenyl), 3.71 (3H, s, CH_3O), 2.66-2.71 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.42-2.47 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.35 (3H, s, CH_3), 4-2.09 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3299 (OH, w), 2217 (CN, s), 1743 (C=O, s), 1590 (C=C, s), 1143 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[4-(cyanophenyl) amino]-5-heptenoate (4g)

Yield 55%; mp : 156; ^{1}H-NMR ($CDCl_3$) 15.43 (1H, s, OH), 7.91 (1H, s, NH), 7.68 (4H, bs, phenyl), 3.70 (3H, s, CH_3O), 2.69-2.74 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.42-2.47 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.05-2.10 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3357 (OH, w), 2217 (CN, s), 1725 (C=O, s), 1646 C=C, s), 1191 (COC, w).

Methyl 6-cyano-5-hydroxy-7-oxo-7-[4-(methylsulfenyl-phenyl)amino]-5-heptenoate (4h)

Yield 52%; mp : 122-123; ^{1}H-NMR ($CDCl_3$) 15.74 (1H, s, OH), 7.42 (1H, s, NH), 7.24-7.39 (4H, m, phenyl), 3.71 (3H, s, CH_3O), 2.66-2.71 (2H, m, $CO\underline{CH_2}CH_2CH_2$), 2.44 (3H, s, SCH_3), 2.42-2.48 (2H, m, $COCH_2\underline{CH_2}CH_2$), 2.04-

2.09 (2H, m, $COCH_2\underline{CH_2}CH_2$); IR (KBr) 3309 (OH, w), 2213 (CN, s), 1743 (C=O, s), 1583 (C=C, s).

Dihydroorotate dehydrogenase assay (Knecht, *et al.*, 1998)

Enzyme assays with the recombinant DHODH were performed after purification at 30°C according to previously described procedures (Knecht, *et al.*, 1996). The oxidation of the substrate dihydroorotate as well as the reduction of the cosubstrate quinone was coupled to the reduction of the chromogen 2,6-dichlorophenolindolphenol (DCIP). The reaction mixture contained either 0.1 mM dihydroubiquinone (Q_D) or 0.1 mM ubiquinone (Q_{10}), 1 mM L-dihydroorotate, 0.06 mM DCIP, 0.1% Triton X-100 in 50 mM Tris-HCl buffer, and 150 mM KCl, at pH 8.0. The reaction was initiated by adding the the enzyme. The loss in absorbance of the blue DCIP was monitored at 600 nm; ε = 18,800 $Lmol^{-1}cm^{-1}$. The enzyme activity in the control assay without Q_D or Q_{10} was approximately 1% of the maximum enzyme activity. This amount was subtracted from the measured activity values. No dye reduction was detected if the control assay was performed in the presence of the enzyme and either Q_D or Q_{10} but in the absence of dihydroorotate. Stock solutions of 10 mM of the test compounds were prepared in dimethylsulfoxide with further dilutions in the buffer taken for the assays. The A771726 was dissolved in the buffer. All solutions were prepared freshly. Dimethylsulfoxide at the stated dilutions did not interfere with the DHODH activity.

RESULTS AND DISCUSSION

The intermediate cyanoacetamide were prepared by activating the cyanoacetic acid with a variety of aromatic amines in the presence of diisopropylcarbodiimide (DIPCDI) (Papageorgiou, *et al.*, 1997). The intermediate cyanoacetamide was then acylated to yield the β-hydroxy enamides **2-4** using sodium hydride and the required acyl halides such as methyl 3-chloro-3-oxopropionate (n = 1), methyl 4-chloro-4-oxobutyrate (n = 2), and methyl 5-chloro-5-oxovalerate (n = 3) (Scheme 1). The resulting compounds contained the α-cyano-β-hydroxy propenamide block, which displaced the methyl group at the 3 position of the metabolite A 771726 with a carboxylalkyl group and bore succinylacetone moiety in the backbone. The compounds were evaluated for their *in vitro* inhibitory activity on human DHODH in order to investigate the relationship between the immunosuppressive activity and the structure. Table I shows the IC_{50} values and the percentage activity. The biological activity of these compounds was initially compared with that of leflunomide (IC_{50} = 97.9 μM) and A 771726 (IC_{50} = 1.08 μM). The IC_{50} values of the **2a**, **3a,** and **4a**,

Table I. Human dihydroorotate dehydrogenase (DHODH) inhibitory potency of the test compounds

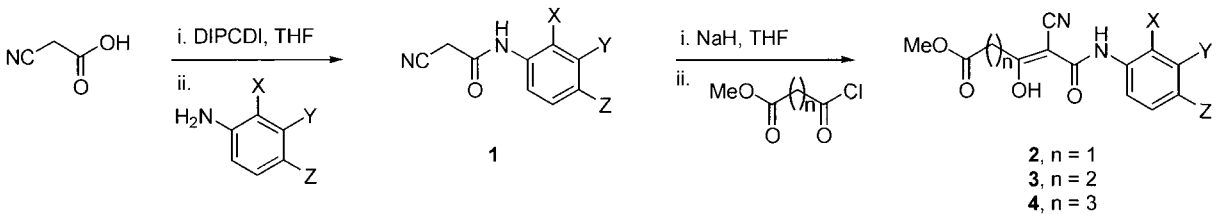
entry	compound	X	Y	Z	n	IC_{50} (μM)[a]
1	**2a**	H	H	CF_3	1	2.6
2	**3a**	H	H	CF_3	2	22.0
3	**3b**	H	CF_3	H	2	60.1±1.3%[d]
4	**3c**	CF_3	H	H	2	[b]
5	**3d**	H	H	Cl	2	35.1±1.8%[d]
6	**3e**	H	Cl	H	2	53.1±6.6%[d]
7	**3f**	H	H	CH_3	2	67.6±2.6%[d]
8	**3g**	H	H	CN	2	59.7±7.1%[d]
9	**3h**	H	H	SCH_3	2	12.1
10	**4a**	H	H	CF_3	3	[c]
11	**4b**	H	CF_3	H	3	79.9±2.6%[d]
12	**4c**	CF_3	H	H	3	[b]
13	**4d**	H	H	Cl	3	[c]
14	**4e**	H	Cl	H	3	[c]
15	**4f**	H	H	CH_3	3	[c]
16	**4g**	H	H	CN	3	98.5±8.5%[d]
17	**4h**	H	H	SCH_3	3	78.1±1.4%[d]
18	A 771726	–	–	–	–	1.08
18	Leflunomide	–	–	–	–	97.9

[a] The human DHODH activity was determined with a chromogen reduction assay using DCIP, dihydroorotate and Q_D at the saturating concentration. The dose response curves for the inhibition of the enzyme were obtained by varying the compound concentrations from 1 nM to 100 μM. The equation was fitted to the initial velocities: $v = V/\{1 + [I]/IC_{50}\}$ ([I] is the inhibitor concentration) in order to determine the concentration causing a 50% inhibition of the enzyme activity (IC_{50}). The results represent an average of at least three experiments.

[b] Insufficient solubility

[c] No inhibition

[d] % Inhibitory activity in the presence of 100 μM



Scheme 1. Synthetic approach to the target molecules

which possessed the same aromatic amide moiety as A 7717126 were found to be dependent on the number of carbons in carboxyalkyl side chain. This means the side chain length controls both the enolic conformation and the polarity of the molecule (Papageorgiou, *et al.*, 1997; Bertolini, *et al.*, 1997). **2a** and **3a** were more potent than leflunomide while **4a** exhibited no inhibitory activity. As expected, the *para* aromatic substituted derivatives showed more potent activity (Table I, entries 2-6). Generally, all compounds with n = 3 such as **4a** had no inhibitory activity. Compared to A77 1726, the designed compounds were less active. However **2a**, **3a**, and **3h** were 4-40 fold more potent than leflunomide.

In conclusion, the inhibitory activities of the carboxylalkyl substituents at the 3 position of β-hydroxy propenamide are less potent than the metabolites of leflunomide while they are more active than leflunomide.

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