Synthesis and Immunosuppressive Activity of Some Side-Chain Variants of Mycophenolic Acid†

Peter H. Nelson,*,‡ Elsie Eugui,§ Ching C. Wang,∥ and Anthony C. Allison§

Syntex Research, 3401 Hillview Ave., Palo Alto, California 94304, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143. *Received June 12, 1989*

The syntheses and immunosuppressive bioassays of 12 side-chain variants of mycophenolic acid (**1a**) are described. The compounds were made either from mycophenolic acid itself or from 5-(chloromethyl)-1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxoisobenzofuran (**3**), a versatile intermediate for the synthesis of diverse side-chain variants. Replacement of the methylated *E* double bond of the natural product with a triple bond, a *Z* double bond, a saturated bond, or a sulfur atom, with overall chain lengths equal to or greater than that of mycophenolic acid, produced compounds devoid of significant activity. Replacement of the side-chain double bond with difluoro, dibromo, or unsubstituted cyclopropane rings also removed most activity. Replacement of the double bond with an allenic linkage yielded a compound with about one-fifth of the immunosuppressive activity of mycophenolic acid. Some possible causes for the unusual specificity of structure and activity are discussed.

Mycophenolic acid (**1a**) is produced by the fermentation of a number of penicillium species.[1] The compound was first described in 1896[2] and the structure was determined in 1952.[3] The compound has been shown to have antifungal,[4] antibacterial,[4] antiviral,[5] and immunosuppressive properties,[6] and it is also effective in several in vitro and in vivo tumor models.[6,7] Mycophenolic acid has been examined in clinical trials against a variety of tumors with little success[8] and also in prolonged trials against psoriasis, in which the compound was effective.[9] Over a considerable period of time, several research groups have attempted to obtain better therapeutic agents based on mycophenolic acid by means of chemical[10] or microbiological[11] modifications or by latentiation.[12] Although mycophenolic acid itself continues to be used in psoriasis on a compassionate basis,[9e] no derivative has yet reached the market.

Mycophenolic acid is a potent inhibitor of inosine monophosphate dehydrogenase (IMPD) and guanosine monophosphate synthetase, with K_i values in the 10^{-8} M range,[7a] and it is therefore an inhibitor of guanosine nucleotide biosynthesis. The in vitro properties can be reversed by the addition of exogenous guanine,[7a] and it is likely that other biological properties are also due to the inhibition of guanosine nucleotide synthesis. Mycophenolic acid is also an inhibitor of soybean lipoxygenase (IC_{50} 55 μm),[13] and it has been hypothesized that this effect may contribute to efficacy in psoriasis, though the antiproliferative effect of the inhibition of purine synthesis seems likely to be more relevant. The high potency of IMPD inhibition by mycophenolic acid is not reflected in low clinical doses (doses in excess of 1 g/day are required in psoriasis[9b,14]) presumably because of rapid clearance of the drug.[15]

In the course of our search for selective nontoxic immunosuppressive agents for use in autoimmune disease, we have examined the effects of inhibitors of purine biosynthesis on lymphocyte responses to mitogenic stimulation. This approach was suggested by the knowledge that lymphocyte responses are reduced in adenosine deaminase deficiency,[16] but are essentially normal in Lesch-Nyhan syndrome (inherited deficiency of hypoxanthine-guanine phosphoribosyl transferase),[17] observations that suggest that lymphocytes are highly dependent on de novo purine biosynthesis.

On the basis of the foregoing and of the observed reduction in intracellular guanosine monophosphate levels in a variety of cell types treated with mycophenolic acid or other IMPD inhibitors,[18,19] we examined the effect of mycophenolic acid on mitogen-induced lymphocyte proliferation. The compound was found to be a potent sup-

† Contribution No. 770 from the Institute of Organic Chemistry.
‡ Syntex Institute of Organic Chemistry.
§ Syntex Institute of Biological Sciences.
∥ University of California, San Francisco, CA.

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Table I. In Vitro Bioassays

compd (*RS*)	inhibn of lymphocyte responses to mitogens: IC_{50}, μm PHA[a]	PWM[a]	SPA[a]	inhibn of IMPD: IC_{50}, μm
1	0.06	0.02	0.06	0.02
2a	3.1	2.8	10	2.6
2b	4.4	>10	>10	3.1
2c	3.8	1.7	4.3	1.1
4a	>10	>10	>10	10.9
4b	>10	>10	>10	22.7
4c	4.8	2.4	7.5	2.6
4d	6.0	6.0	>10	2.5
4e	4.8	4.8	4.8	1.8
4f	>10	>10	>10	NT[b]
4g	0.35	0.35	0.15	1.2
4h	2.5	4.3	1.6	0.94
4i	>10	>10	>10	1.8

[a] PHA, phytohaemmagglutinin; PWM, pokeweed mitogen; SPA, staphylococcus protein A. [b] Not tested.

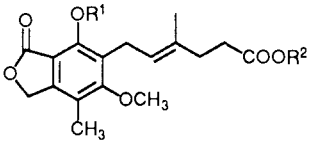
pressor of the responses of both T and B lymphocytes to mitogens, at concentrations of 10^{-7} M or less. Since mycophenolic acid is relatively innocuous in experimental animals (the LD_{50} in rats and mice is 452 and 1917 mg/kg per day, respectively[7c]), we have attempted to synthesize analogues more effective than the parent compound.

Previous work, in which analogues of mycophenolic acid were tested for antimitotic[10] or antitumor[6c] activity, had shown that biological activity was lost if significant modifications were made to the structure. In view of the fact that hydrogenation of the side-chain double bond was known to cause marked loss of antimitotic potency,[10] we synthesized a group of compounds that retained π-electron character in the appropriate positions on the side chain relative to the nucleus and the terminal carboxyl group.

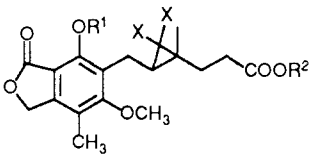
Chemistry

The cyclopropane analogues **2a–c** were prepared from appropriately protected derivatives of mycophenolic acid. Attempts to add difluorocarbene, generated by thermolysis of sodium chlorodifluoroacetate, to mycophenolic acid itself, apparently gave no reaction[20] so a protected substrate was examined. The corresponding reaction of methyl *O*-methylmycophenolate[11] (**1b**) gave a good yield of the adduct **2d**, and selective demethylation using boron trichloride,[21] followed by alkaline hydrolysis of the ester, then afforded **2a**. The dibromocyclopropane **2b** was also prepared from a doubly protected mycophenolic acid; in this case both the hydroxyl groups were protected as MEM ethers. Dibromocarbene was generated under phase-transfer conditions from bromoform and aqueous sodium hydroxide. The allenic derivative **4g** was prepared from the dibromocyclopropane with use of the $CrCl_3$/LAH reagent of Hiyama et al.[22] Since yields were low in the initial preparation of the dibromocyclopropane adduct, different protecting groups were used when larger quantities were required (see the Experimental Section). The unsubstituted cyclopropane **2c** was prepared by means of a modified Simmon-Smith reaction[23] on mycophenolic acid itself.

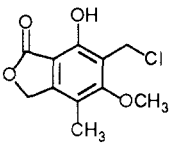
The remaining analogues were prepared from the (chloromethyl)phenol **3**,[24] by means of displacement reactions with 3-mercaptopropionic acid, 4-mercaptobutyric acid, and the bis(bromomagnesium) salts of pent-4-ynoic and hex-5-ynoic acids. These reactions proceeded rapidly and in good yields, presumably because of elimination of hydrogen chloride from **3a** to give a highly reactive *o*-quinone methide. The procedure is a facile route to side-chain variants of mycophenolic acid and, if it was justified by biological results, could be used to prepare a wide range of analogues by means of nucleophilic displacement of the chloride. The acetylenic products of the latter reactions were partially or completely hydrogenated to produce the *Z* olefins and the saturated side-chain analogues.



1a: $R^1 = R^2 = H$
b: $R^1 = R^2 = CH_3$
c: $R^1 = R^2 = CH_2O(CH_2)_2OCH_3$
d: $R^1 = CH_2O(CH_2)_2OCH_3$; $R^2 = CH_3$



2a: $R^1 = R^2 = H$; X = F
b: $R^1 = R^2 = H$; X = Br
c: $R^1 = R^2 = X = H$
d: $R^1 = R^2 = CH_3$; X = F
e: $R^1 = H$; $R^2 = CH_3$; X = F
f: $R^1 = CH_2O(CH_2)_2OCH_3$; $R^2 = CH_3$; X = Br



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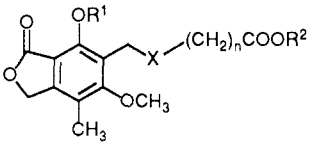

4a: $R^1 = R^2 = H$; X = S; $n = 2$
b: $R^1 = R^2 = H$; X = S; $n = 3$
c: $R^1 = R^2 = H$; X = C≡C; $n = 2$
d: $R^1 = R^2 = H$; X = C≡C; $n = 3$
e: $R^1 = R^2 = H$; X = (*Z*)—CH=CH; $n = 2$
f: $R^1 = R^2 = H$; X = (*Z*)—CH=CH; $n = 3$
g: $R^1 = R^2 = H$; X = CH=C=C(CH$_3$); $n = 2$
h: $R^1 = R^2 = H$; X = $(CH_2)_2$; $n = 2$
i: $R^1 = R^2 =$H; X = $(CH_2)_2$; $n = 3$
j: $R^1 = H$; $R^2 = CH_3$; X = CH=C=C(CH$_3$); $n = 2$

Discussion

All the analogues were assayed for their ability to inhibit mitogen-induced human lymphocyte proliferation. Eleven of the compounds were also tested for inhibition of lymphoid-derived IMPD. The results are shown in Table I. As stated earlier, mycophenolic acid is a potent inhibitor of the blastic transformation, with IC_{50} values in the 10^{-8} M range. It exhibits similar activity against different lymphocyte subsets; thus responses to PHA, which stimulates T cells,[25] and PWM and SPA, which respectively

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Table II. Effect of **1a** and **4g** on in Vivo Antibody Response to SRBC in Mice

treatment,[a] days 0 to 4	no. of animals	po dose, mg/kg per day	PFC/spleen[b]	% inhibn	PPM[c]	% inhibn	WBC/spleen × 10^6
			Experiment 1				
vehicle	6		117 416		751		158
1a	6	25	36 027[d]	69	264[d]	65	137
4g	6	25	96 583	18	630	16	155
4g	6	40	99 333	15	583	22	169
			Experiment 2				
vehicle	7		84 142		544		153
1a	6	25	45 333[d]	46	308[d]	44	142
4g	6	50	112 416	−33	657	−20	172
4g	6	100	17 000[d]	80	114[d]	79	154

[a] All animals received 1 × 10^6 SRBC ip on day 0. [b] Figures are means of the total number of PFC/spleen per group (quadruplicate samples from each animal). [c] Plaques per million (PPM) are the total number of plaques in the spleen divided by 10^{-6} times the total number of nucleated cells. [d] $p < 0.05$.

activate T-dependent[25] and T-independent B cells,[26] are inhibited similarly.

Previous workers have shown that the biological activities of mycophenolic acid are unusually sensitive to even small structural changes. Replacement of the methoxy group by an ethoxy, of the aromatic methyl by hydrogen, or of the phenolic hydroxyl by methoxy all resulted in compounds with little or no activity.[10] The terminal carboxyl group was found to be necessary for activity, and compounds with either longer or shorter side chains were biologically inactive. Of more relevance to the present work was the finding that dihydromycophenolic acid, produced by catalytic hydrogenation of the side-chain double bond, has less than one-tenth of the potency of mycophenolic acid as an inhibitor of mouse fibroblast mitosis.[10] The present series of compounds was synthesized in the hope that the *E* double bond could be replaced by *Z* olefinic, acetylenic, or allenic moieties, or that the π character of the double bond could be effectively simulated by a sulfur atom or a cyclopropane ring.

The results shown in Table I clearly show that our expectations were not fulfilled. Although many of the analogues inhibited both lymphocyte responses and IMPD at micromolar concentrations, none had the potency of the parent compound. The two assays showed a reasonable correlation in some cases but not in others. Thus the thio analogues **4a** and **4b** were almost inactive in both assays, and the dibromocyclopropane compound **2b** was only slightly more potent. Both the difluoro-substituted and unsubstituted cyclopropanes **2a** and **2c** were more active than the dibromocyclopropane, presumably because the large size of the latter was even less compatible with the receptor site. However, if size and the presence of π or d electrons was a criterion for activity, then the thio compounds would be predicted to show higher potency. Rationalizations are also difficult for the analogues containing olefinic or acetylenic groups. Compound **4e** represents a minimal change in structure from the parent mycophenolic acid: removal of a methyl group and reversal of the double-bond stereochemistry. Yet the compound has about 1% of the potency of **1a** and is apparently less active than the corresponding dihydro derivative **4h**. The acetylenic analogues **4c** and **4d** are also only minimally active, and it is only in the allenic compound **4g** that the ability to inhibit lymphocyte responses even approaches that of the parent. This compound is from 0.2 to 0.4 times as potent as **1a**, depending on the mitogen. The relative potency for IMPD inhibition is only 0.017, however, and obviously in this instance the two assays do not correlate well. The allene analogue does show weak in vivo immunosuppressive activity in the Jerne plaque assay,[27] reflecting the modest potency in the mitogen assay rather than the very low potency for inhibition of IMPD. The compound was inactive at oral doses of 25, 40, and 50 mg/kg in the mouse, but significantly reduced the number of plaque-forming cells (PFC) in the spleen after four daily doses of 100 mg/kg. Mycophenolic acid produced significant immunosuppression at a dose of 25 mg/kg. None of the other compounds in Table I was active. The rough correlation in potencies for **1a** and **4g** in the mitogen and in vivo assays, contrasted to the lack of correlation for IMPD inhibition, may indicate that the allene analogue is a more potent inhibitor of guanosine monophosphate synthetase than of IMPD, though we have no evidence to support such a hypothesis. Numerical correlations between IMPD inhibition and immunosuppression have not been reported in other series, although the immunosuppressant and antiviral properties of ribavirin, bredinin, and tiazafurin are ascribed to IMPD inhibition.[28,29]

Mycophenolic acid and inosine monophosphate do not appear to be sufficiently similar in structure that **1a** could act as a substrate analogue in the inhibition of IMPD. This empirical conclusion, supported by the stringent requirements for activity in the series, has been verified by kinetic data which show mycophenolic acid to be an uncompetitive inhibitor of IMPD from the intracellular protozoan *Eimeria tenella*[30] and from the anaerobic protozoan *Tritrichomonas foetus*.[31] Mycophenolic acid was shown to bind to the enzyme–product (XMP) complex but not to the enzyme–IMP complex. A proposal that the mycophenolic acid phenolic hydroxyl groups adds nucleophilically to the enzyme–XMP adduct, so as to give a tetrahedral intermediate that resembles that required for hydride transfer to NAD, following the addition of water to IMP, has been advanced.[30] Although this model would rationalize the observed inactivity of the methyl ether of mycophenolic acid, it does not explain why relatively minor structural changes far removed from the phenolic group cause almost complete loss of activity. Results cited or described herein indicate the presence on

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IMPD of a binding site, different from the substrate binding site, to which only mycophenolic acid can bind with high affinity. Binding to this site may cause an allosteric modification of the enzyme that prevents release of the product XMP. Logically it seems unlikely that no active analogues can be made, but the results indicate that changes which can be made are likely to be minimal. Mycophenolic acid itself is rapidly inactivated by glucuronidation at the hydroxyl group, a process thought to account for its relatively low in vivo potency. An active analogue, differing only slightly from the parent molecule, would probably be similarly metabolized; thus the prospects for obtaining a more effective agent by structural modification do not appear to be promising.

Experimental Section[32]

1-(2-Carboxyethyl)-2,2-difluoro-3-[(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-methyl]-1-methylcyclopropane (2a). Anhydrous Na_2CO_3 (2.38 g, 23 mmol) was added to ClF_2CCOOH (6.02 g, 46 mmol) in diglyme (70 mL). The mixture was stirred and warmed to ca. 40 °C for 1.5 h, and then 35 mL of the diglyme was removed under vacuum (T <60 °C). Methyl O-methylmycophenolate (1b) (0.7 g, 2 mM) was dissolved in diglyme (distilled from KOH and then from Na) (10 mL), and the solution was stirred vigorously in a 160 °C oil bath. A 10-mL portion of the sodium chlorodifluoroacetate solution was then added in one lot; the mixture was heated for 30 min and then a second 10-mL portion was added. After a further 15 min at 160 °C the mixture was cooled and added to water. The product, a dark oil, was extracted with EtOAc and chromatographed on silica gel (3:1 hexane–acetone) to give 0.73 g (89%) of **2d** as an oil: NMR[33] δ 1.17 (nm, 3 H, cyclopropyl-CH_3), 3.65 (s, 3 H) and 3.80 (s, 3 H) (2 $ArOCH_3$). **2d** (500 mg, 1.26 mmol) was dissolved in CH_2Cl_2 at 0 °C and 1.0 M BCl_3 in CH_2Cl_2 (5.0 mL, 5 mmol) was added. The mixture was left at room temperature for 3 h and then added to water. The product was isolated by evaporation of the dried organic phase and was chromatographed on silica gel (50 g, 400:400:1 Et_2O–hexane–AcOH) to afford 107 mg, 22% of the phenolic methyl ester **2e** as a gum: NMR[33] δ 1.18 (nm, 3 H, cyclopropyl-CH_3), 3.80 (s, 3 H, $ArOCH_3$), 4.11 (s, 3 H, $COOCH_3$). This material was dissolved in 1,2-dimethoxyethane (2 mL) and a solution of $LiOH·H_2O$ (100 mg, 2.38 mM) in water (1 mL) was added. After 15 min the solution was added to water, washed with Et_2O, and acidified with dilute HCl. The product **2a** was extracted with Et_2O and recrystallized from Et_2O–hexane (73 mg, 71%): mp 122–3 °C; NMR[33] δ 1.18 (nm, 3 H, cyclopropyl-CH_3), 3.80 (s, 3 H, $ArOCH_3$). Anal. ($C_{18}H_{20}F_2O_6$) C, H.

1-(2-Carboxyethyl)-3,3-dibromo-2-[(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-methyl]-1-methylcyclopropane (2b). Mycophenolic acid (2.0 g, 6.25 mmol) and Et_3N (1.39 g, 13.8 mmol) were dissolved in a mixture of THF (10 mL) and CH_2Cl_2 (50 mL). The solution was cooled to 0 °C and MEM chloride (1.71 g, 13.8 mmol) was added. After 1 h at 0 °C and 24 h at room temperature, aqueous $NaHCO_3$ was added and the organic phase was dried and evaporated. The residue was chromatographed on silica gel (100 g, 1:1 Et_2O–hexane) to give **1c** (0.60 g, 19%) as an oil: NMR[33] δ 1.83 (s, 3 H, CH_3), 3.40 (s, 6 H, OCH_3), 3.78 (s, 3 H, $ArOCH_3$).

Compound **1c** (496 mg, 0.98 mmol) was dissolved in $CHBr_3$ (2.51 g, 9.8 mmol) and EtOH (0.02 mL). Tetrabutylammonium bromide (3 mg) and 50% aqueous NaOH (0.43 mL) were added. The mixture was stirred for 24 h. Water and CH_2Cl_2 were then added, and the aqueous phase was washed with Et_2O and acidified with dilute HCl and extracted with Et_2O. The extract was stirred overnight with dilute HCl to remove the MEM ether (the MEM ester was removed during the carbene addition reaction), then dried, and evaporated. The residue (300 mg) was chromatographed on silica gel (40:8:1 CH_2Cl_2–MeOH–NH_4OH) to afford **2b** (67 mg, 14%): mp 134–7 °C (acetone–hexane); NMR[33] δ 1.27 (s, 3 H, cyclopropyl-CH_3), 1.60 (m, 1 H, cyclopropyl H), 2.55–2.65 (m, 1 H) and 2.85–2.95 (m, 1 H) ($ArCH_2$). Anal. ($C_{18}H_{20}Br_2O_6$) C, H.

1-(2-Carboxyethyl)-3-[(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)methyl]-1-methylcyclopropane (2c). Zinc dust (3.3 g, 50.7 mmol) and CuCl (3.0 g, 30 mmol) were mixed together and then refluxed with stirring in THF (30 mL) for 30 min. The mixture was cooled to 0 °C and CH_2I_2 (10 g, 37.3 mmol) was added. The mixture refluxed spontaneously, and reflux was continued for 15 min. Compound 1 (0.5 g, 1.56 mM) in THF (15 mL) was added and the reaction mixture was refluxed with stirring for 2 h. The cooled mixture was diluted with Et_2O (50 mL) and decanted. The organic solution was extracted with dilute aqueous KOH, and the extract was acidified and extracted with EtOAc. The EtOAc was dried and evaporated, and the residue was chromatographed on silica gel (50 g, 40:12:1 CH_2Cl_2–MeOH–NH_4OH) to afford 210 mg (40%) of **2c**: mp 130–5 °C (EtOAc–hexane). Anal. ($C_{18}H_{22}O_6$) C, H. The mass spectrum showed a molecular ion at m/e 334 (5%) and ions at m/e 316 (10) and 207 (100). NMR[33] δ 0.20 (m, 1 H) and 0.40 (m, 1 H) (cyclopropane CH_2), 0.9 (m, 1 H, cyclopropane CH), 1.16 (s, 3 H, cyclopropyl-CH_3).

2-Carboxyethyl (1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)methyl Sulfide (4a). 3-Mercaptopropionic acid (197 mg, 1.8 mmol) was added to a solution of **3** (300 mg, 1.23 mmol) and diisopropylethylamine (640 mg, 4.96 mmol) in THF (8 mL). After 16 h the mixture was added to dilute HCl–EtOAc. The organic phase was extracted with aqueous $NaHCO_3$, and the extract was washed with EtOAc and then acidified with dilute HCl. The acidified solution was then extracted with EtOAc, and the extract was dried and evaporated to give 290 mg of a solid, which was recrystallized from aqueous MeOH to afford **4a** (181 mg, 47%): mp 153–5 °C; NMR[33] δ 2.5–2.8 (m, 4 H, $CH_2)_2$), 3.75 (s, 2 H, $ArCH_2S$). Anal. ($C_{14}H_{16}O_6S$) C, H, S.

The higher homologue, **3-carboxypropyl (1,3-dihydro-4-hydroxy-6-methoxy-3-oxo-5-isobenzofuranyl)methyl sulfide** (4b) was prepared in 37% yield with use of 4-mercaptobutyric acid and by the same procedure: mp 116–8 °C (aqueous MeOH); NMR[33] δ 2.49 (t, J = 8, 2 H, CH_2COOH), 2.66 (t, J = 8, 2 H, CH_2S), 3.81 (s, 2 H, $ArCH_2S$). Anal. ($C_{15}H_{18}O_6S$) C, H, S.

6-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-hexynoic Acid (4c). Ethereal EtMgBr (2.6 M; 1.43 mL, 3.7 mmol) was added to a solution of 4-pentynoic acid (196 mg, 2 mmol) in dry THF (10 mL) at 0 °C. The solution was stirred at 0 °C for 1.5 h, and then cuprous cyanide (20 mg, 0.25 mM) was added. The mixture was then stirred at room temperature for 30 min, and then a suspension of **3** (218 mg, 0.90 mmol) in THF (2 mL) was added. After 16 h the mixture was added to dilute HCl and extracted with EtOAc. The organic phase was extracted with dilute aqueous KOH. The basic extract was washed with Et_2O and then acidified with dilute HCl, and the crude semicrystalline product (270 mg) was isolated by extraction with EtOAc. The product was purified by preparative TLC (3 × 20 cm × 40 cm × 1 mm silica gel plates, 50:1 Et_2O–AcOH) to give **4c** (111 mg, 41%): mp 157–9 °C (ether–hexane); NMR[33] δ 2.48 (m, 4 H, CH_2CH_2), 3.53 (s, 2 H, $ArCH_2$); MS m/e 304 (48) M^+. Anal. ($C_{16}H_{16}O_6$) C, H.

With use of 5-hexynoic acid and the same procedure, **7-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)hept-5-ynoic acid (4d)** was prepared in 19% yield: mp 124–6 °C (EtOAc); NMR[33] δ 2.45 (t, J = 8, 2 H, CH_2COOH), 3.56 (t, J = 2, 2 H, $ArCH_2$). Anal. ($C_{17}H_{18}O_6$) C, H.

(Z)-6-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-hexenoic Acid (4e). $Pd/CaCO_3$ (5%), poisoned with lead (100 mg) was prehydrogenated in EtOAc (30 mL) and quinoline (0.5 mL) for 15 min. A solution of **4c** (304 mg, 1 mmol) in EtOAc (5 mL) was added. An uptake of 21.4 mL (theoretical uptake 22.4 mL) occurred during 25 min, and then uptake stopped. The solution was filtered and washed with dilute

(32) Melting points are uncorrected. All reactions were performed under dry nitrogen. NMR spectra were recorded in $CDCl_3$ on a Bruker WM 300 NMR spectrometer, with TMS as internal reference, and were in accord with the assigned structures. Relevant resonances (ppm) and coupling constants (hertz) are quoted in the text.

(33) Unless otherwise indicated, all compounds had resonances at 2.14–2.17 ($ArCH_3$), 3.78–3.80 ($ArOCH_3$), and 5.16–5.22 (phthalide CH_2).

HCl and then dried and evaporated. The residue was recrystallized to afford 190 mg (62%) of **4e**: mp 155–6 °C (acetone–hexane); NMR[33] δ 2.5–2.65 (m, 4 H, CH_2CH_2), 3.45 (d, J = 6, 2 H, $ArCH_2$), 5.35–5.50 (m, 2 H, CH=CH). Anal. ($C_{16}H_{18}O_6$) C, H.

Similarly, Lindlar hydrogenation of **4d** produced (*Z*)-7-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)hept-5-enoic acid (**4f**) in 71% yield: mp 119–120 °C (EtOAc); NMR[33] δ 2.34 (m, 2 H, CH_2C=C), 2.42 (t, J = 7, 2 H, CH_2COOH), 3.42 (d, J = 6, 2 H, $ArCH_2$), 5.35–5.55 (m, 2 H, CH=CH). Anal. ($C_{17}H_{20}O_6$) C, H.

(±)-7-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methylhepta-4,5-dienoic Acid (4g). The allene function was generated from the corresponding dibromocyclopropane; however, since the initial preparation of this functional group (see above) gave low yields, an alternative route was developed.

(a) To a solution of methyl mycophenolate **1b** (15.0 g, 45 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C was added Et_2NPr^i (7.23 g, 56 mmol) and then, over 10 min, MEM chloride (6.96 g, 56 mmol). The mixture was left at room temperature for 4 h, then added to water, washed with dilute HCl and dilute aqueous KOH, dried, and evaporated to afford 18.9 g (96%) of **methyl (*E*)-6-[(1,3-dihydro-6-methoxy-4-[(methoxyethoxy)methoxy]-7-methyl-3-oxo-5-isobenzofuranyl)methyl]-4-methyl-4-hexenoate (1d)**: mp 119–121 °C (ether–hexane); NMR[33] δ 1.67 (s, 3 H, CH_3C=C), 2.4–2.5 (m, 4 H, $(CH_2)_2$), 3.37 (s, 3 H, OCH_3), 3.68 (s, 3 H, $COOCH_3$), 3.5–4.0 (m, 8 H, CH_2O and $ArCH_2$), ca. 5.1 (m, 1 H, C=CH). Anal. ($C_{22}H_{30}O_8$) C, H.

(b) The above product (18.8 g, 43 mmol) was dissolved in $CHBr_3$ (38 mL), and tetrabutylammonium bromide (38 mg), methanol (0.3 mL), and 50% aqueous NaOH (13 mL) were added. The mixture was stirred for 48 h and then added to water and CH_2Cl_2. The aqueous solution was washed with water and then acidified with dilute HCl and extracted with EtOAc. The extract was dried and evaporated to yield 22.4 g of an oil. This material was dissolved in DMF (400 mL), and MeI (30 mL) and Na_2CO_3 (30 g) were added. The mixture was stirred for 3 h and then decanted and added to water. The aqueous solution was extracted with Et_2O, and the extract was dried and evaporated to give 22.6 g of an oil. This material was chromatographed on silica gel (2 kg, 2:1 then 1:1 hexane–Et_2O) to afford 9.7 g (37%) of **2-(2-carbomethoxyethyl)-1,1-dibromo-3-[[1,3-dihydro-4-methoxy-6-[(methoxyethoxy)methoxy]-7-methyl-3-oxo-5-isobenzofuranyl]methyl]-1-methylcyclopropane (2f)** as an oil: NMR[33] δ 1.25 (s, 3 H, cyclopropyl–CH_3), 3.35 (s, 3 H, OCH_3), 3.67 (s, 3 H, $COOCH_3$).

(c) The above product (9.7 g) was dissolved in MeOH (115 mL), and concentrated HCl (6 mL) was added. The mixture was stirred for 5.5 h, during which time the product precipitated. The solid was isolated by filtration, dried, and recrystallized to afford **1-(2-carbomethoxyethyl)-2,2-dibromo-3-[(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-methyl]cyclopropane (2g)** (3.11 g, 38%): mp 218–220 °C (aqueous MeOH); NMR[33] δ 1.27 (s, 3 H, cyclopropyl–CH_3), 2.37 (t, J = 7, 2 H, CH_2COOH), 3.63 (s, 3 H, $COOCH_3$). Anal. ($C_{19}H_{22}Br_2O_6$) C, H, Br.

(d) Allene formation:[22] LAH (219 mg, 5.8 mmol) was added to a suspension of $CrCl_3$ (2.03 g, 12.8 mmol) in THF (30 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h and then cooled to −80 °C. Vacuum was applied, and the cooling bath was removed and the mixture was stirred vigorously as the THF evaporated to leave a black solid. Dry DMF (20 mL) and then a solution of **2g** (1.4 g, 2.71 mmol) in DMF (5 mL) were added. The reaction was then heated in a 65 °C oil bath for 2 h, then cooled, added to water, and extracted with EtOAc. The extract was dried and evaporated, and the product, a green oil, was chromatographed on silica gel (20 g, 2:1 hexane–Et_2O) to yield **(±)-methyl 7-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methylhepta-4,5-dienoate (4j)** (680 mg, 76%): mp 85–7 °C (hexane); NMR[33] δ 1.63 (s, 3 H, CH_3C=), 2.3–2.4 (m, 4 H, CH_2CH_2), 3.3–3.4 (m, 2 H, CH_2C=), 5.25–5.33 (m, 1 H, CH=C). Anal. ($C_{19}H_{22}O_6$) C, H.

(e) Hydrolysis: A solution of **4j** (250 mg, 0.72 mmol) in THF (3.7 mL) was diluted with water (3.7 mL) and then a solution of $LiOH·H_2O$ (63 mg, 1.5 mM) in water (1 mL) was added. After 3 h the solution was acidified with dilute HCl and then extracted with Et_2O. The extract was dried and evaporated to give 231 mg (97%) of **4g**: mp 82–4 °C (Et_2O–hexane); NMR[33] δ 1.63 (d, J = 2, 3 H, CH_3C=C), 3.35 (d, J = 7, 2 H, $ArCH_2$), 5.25–5.35 (m, 1 H, CH=C). Anal. ($C_{18}H_{20}O_6$) C, H.

6-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)hexanoic Acid (4h). A solution of **4c** (200 mg, 0.66 mmol) was hydrogenated for 1 h at atmospheric pressure in EtOAc (20 mL) containing 5% Pd/C (100 mg). The solution was filtered and evaporated, and the residue was recrystallized from acetone/hexane to give 134 mg (66%) of **4h**: mp 128–130 °C; NMR[33] δ 2.36 (t, J = 7, 2 H, CH_2COOH), 2.66 (t, J = 8, 2 H, $ArCH_2$). Anal. ($C_{16}H_{20}O_6$) C, H.

Similarly, catalytic hydrogenation of **4d** gave 75% of **7-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)heptanoic acid** (**4i**): mp 108–110 °C (Et_2O–hexane); NMR[33] δ 2.35 (t, J = 8, 2 H, CH_2COOH), 2.65 (t, J = 8, 2 H, $ArCH_2$). Anal. ($C_{17}H_{22}O_6$) C, H.

Testing for in Vitro Effects on Human PBL Responses to Mitogens. Human PBL were separated from heparinized whole blood from a single, healthy, drug-free donor by density gradient centrifugation in Ficoll-Paque (Pharmacia). After washing, 2×10^5 cells/well were cultured in microtiter plates with RPMI-1640 medium (Gibco) supplemented with 5% fetal calf serum (FCS, Gibco), 50 units/mL penicillin, 50 μg/mL streptomycin, and 2 μM L-glutamine. To evaluate differential effects on T- and B-lymphocytes, the following mitogens were used: phytohemagglutinin (PHA, Sigma) at 10 μg/mL, pokeweed mitogen (PWM, Sigma) at 20 μg/mL, and *staphylococcus* protein A bound to sepharose (SPA, Sigma) at 2 mg/mL (14 μg/mL of protein A). Compounds were tested at concentrations of 10^{-8}–10^{-5} M by addition to the culture at time 0. All compounds were first solubilized in dimethyl sulfoxide at 10^{-2} M concentration and were further diluted in RPMI-1640 medium to achieve the final concentration. Cultures were set up in qudruplicate in 96-well plates and incubated at 37 °C in an atmosphere of air containing 5% CO_2 (100% humidity) for 72 h. A pulse of 0.5 μCi/well of [^{3}H]thymidine (^{3}H-Tdr, New England Nuclear, specific activity = 6.7 Ci/mmol) was added for the last 6 h. Cells were collected on glass fiber filters (PHD, Cambridge Technology) with an automatic harvester (PHD), and radioactivity was measured in a liquid scintillation spectrophotometer, after addition of 4 mL of scintillation fluid (Aquasol, New England Nuclear) to each vial. The 50% inhibitory concentration (IC_{50}) for mitogenic stimulation was determined graphically.

Test for in Vivo Effects on Antibody Production. A modification of the Jerne hemolytic plaque assay[27] was used. Groups of six to seven adult C57B1/6 mice (Bantin and Kingman, Fremont CA) were sensitized with 1×10^8 sheep red blood cells (SRBC) ip and simultaneously treated orally with the test compound, suspended in a vehicle consisting of 0.9% NaCl, 0.5% sodium (carboxymethyl)cellulose, 0.4% polysorbate 80, 0.9% benzyl alchol, and 97.3% water. Four daily doses of test compounds were administered. Animals in the control group received the same volume of vehicle. On day 4 post SRBC inoculation, animals were sacrificed and spleens were dispersed in loose Ten Broeck homogenizers, the number of nucleated cells (WBC) was determined, and the spleen cell suspension was mixed with SRBC, guinea pig complement, and agar solution at 0.5% concentration. Aliquots of the above mixture (0.1 mL) were dropped in four separate quadrants of a petri dish and covered with cover slips. After a 2-h incubation at 37 °C, areas of hemolysis around plaque-forming cells were counted with a dissecting microscope. Total WBC/spleen, PFC/spleen, and PFC/10^6 WBC (ppm) were calculated for each mouse spleen. Means of each treatment group were then compared with the vehicle-treated control, and Student's t test was applied to calculate p values.

Inhibition of IMP Dehydrogenase. Approximately 1 mL of packed acute human lymphoblastic leukemia (MOLT-4)[34] cells were suspended in 1 mL of pH 7.4 buffer (50 mM potassium phosphate, 10 mM β-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride). The suspension was frozen and thawed

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five times and was then centrifuged at 3000 rpm for 2 min. The slightly cloudy upper phase was removed and centrifuged at 16000 rpm (ca. 31000g) for 45 min. The supernatant (1.6 mL) was separated into small aliquots and kept at -80 °C. Enzyme activity was assayed as described in ref 35. In a final volume of 100 μL, IMPD (4 μL of the above preparation) was measured in 50 mM potassium phosphate buffer, pH 7.4, 10 μM [^{14}C]IMP (50 mCi/mM), 0.3 mM NAD, 0.1 M KCl, and 1 mM EDTA. The reactions were stopped after 25 min incubation at 37 °C by addition of 4 μL of 5 N $HClO_4$. After cooling in ice for 15 min, the protein precipitate was removed by centrifugation. The aqueous supernatant was transferred to a new tube and neutralized by mixing with an equal volume of Freon (Du Pont) and trioctylamine (1:1). The aqueous phase was removed, and the nucleotides were analyzed by HPLC, using an Ultrasil AX (10 μm) column over a salt gradient;[36] the amounts of both IMP and XMP were determined by a radioactive flow detector. Three microliters of unlabeled IMP (10 mM) and XMP (10 mM) solutions were added as carriers before HPLC analysis. Compounds were dissolved in DMSO (4 mg/mL); each compound was tested at five different concentrations from 0.02 to 5.0 μg/mL, and IC_{50} values were determined graphically.

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Renin Inhibitors Containing Isosteric Replacements of the Amide Bond Connecting the P_3 and P_2 Sites

J. S. Kaltenbronn,* J. P. Hudspeth, E. A. Lunney, B. M. Michniewicz, E. D. Nicolaides, J. T. Repine, W. H. Roark, M. A. Stier, F. J. Tinney, P. K. W. Woo, and A. D. Essenburg

Departments of Chemistry and Pharmacology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105. *Received March 16, 1989*

Renin inhibitors having 13 different isosteres connecting the P_3 and P_2 positions have been prepared. Synthetic routes and in vitro activity exhibited by these compounds are discussed. The two most potent compounds, **47** and **48**, contained the hydroxyethylene isostere, Ψ[$CHOHCH_2$], and had IC_{50} values of 61 and 22 nM, respectively.

The success of angiotensin converting enzyme (ACE) inhibitors in the treatment of hypertension[1a,b] has demonstrated that interrupting the biochemical cascade

angiotensinogen $\xrightarrow{\text{renin}}$ angiotensin I $\xrightarrow{\text{ACE}}$ angiotensin II

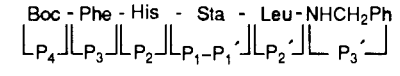
can lead to a lowering of blood pressure. This result has prompted our group and others[2] to seek agents that interrupt this cascade at an earlier stage by inhibition of the action of renin on angiotensinogen.

In recent years increasing interest has been shown in the concept of isosteric replacement of amide bonds in biologically active peptides. Inherent in this concept is the postulate that it might be possible to modify one or more amide bonds in peptides such that conformation and binding are maintained, but enzymatic hydrolysis is prevented. Initial successes utilizing this concept have been reported by Spatola,[3] who used the methylenethio isostere, Ψ[CH_2S], as an amide replacement in enkephalin analogues, and Szelke,[4] who prepared renin inhibitors having both the methyleneamino, Ψ[CH_2NH], and hydroxyethylene, Ψ[$CHOHCH_2$], isosteres at the scissile Leu-Val amide bond in the 6-13 octapeptide derived from angiotensinogen. Reports from our laboratories have described modified di- and tripeptides derived from the C-terminal portion of oxytocin and vasopressin as possible cognition-activating agents.[5]

Table I. Peptide Bond Isosteres Prepared

Ψ[CH=CH]	Ψ[$COCH_2$]
Ψ[CHCHO]	Ψ[CH_2NH]
Ψ[CH_2CH_2]	Ψ[CH_2NOH]
Ψ[CHOHCHOH]	Ψ[CH_2S]
Ψ[CHOHCH=CHCO]	Ψ[CH_2SO]
Ψ[CHOHCHOHCHOHCO]	Ψ[CH_2SO_2]
Ψ[$CHOHCH_2$]	

As one aspect of our renin inhibitor strategy, we chose to prepare modified compounds based on the potent renin inhibitor (I) reported by Bock.[6,7]



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While a number of other groups[4,8a-c,9,10] have described

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