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TETRAZOLE-SUBSTITUTED UREAS AS INHIBITORS OF ACYL-CoA:CHOLESTEROL *O*-ACYLTRANSFERASE (ACAT). A NOVEL PREPARATION OF UREAS FROM WEAKLY NUCLEOPHILIC AMINES

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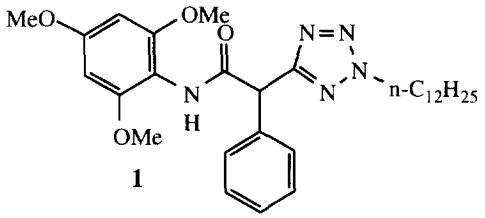
Abstract: A novel series of tetrazole-substituted ureas **2** were prepared from weakly nucleophilic amines using a new coupling method. The ureas were found to potently inhibit liver ACAT in vitro and lower total serum cholesterol in vivo. A comparison of urea **2b** and the anti-atherosclerotic CI-976 in a long-term model of atherosclerosis indicates the importance of inhibiting arterial ACAT for reducing lesion size.

A recent report[1] from our laboratories described the tetrazole-substituted amide **1** as a novel inhibitor of the enzyme acyl-CoA:cholesterol *O*-acyltransferase (ACAT).[2] Compared to CI-976,[3] amide **1** was found to more potently inhibit ACAT in vitro and to more efficaciously lower total plasma cholesterol in vivo.[4] Since the urea functionality has proved to be an active bioisosteric replacement for amide,[5] and since it also eliminates potential problems with chirality of the amide, we decided to replace the α-carbon of **1** with nitrogen to yield a series of tetrazole-substituted ureas **2**. In this paper, we report on the biological activities of the ureas **2**, including bioavailability and anti-atherosclerotic activity, and on a new method for preparing these ureas from non-nucleophilic secondary amines.



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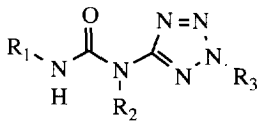

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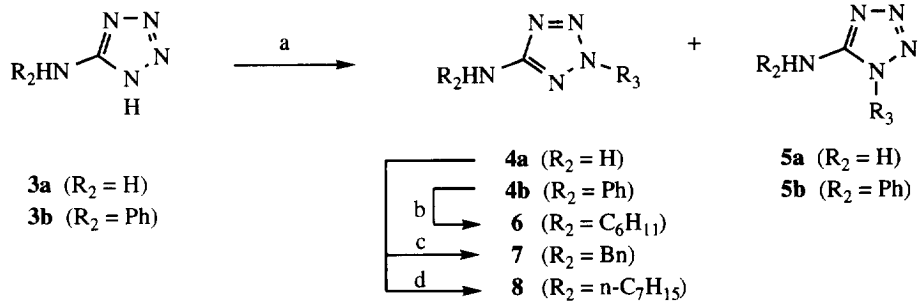
Chemistry: The secondary amines **4b**, **6**, **7**, and **8** used in the synthesis of the ureas were prepared by the synthetic route outlined in Scheme 1. The amino tetrazoles **3a** or **3b** were alkylated with alkyl bromide R_3Br by stirring with triethylamine in refluxing acetonitrile to give the regioisomeric tetrazoles **4** and **5**, which can be separated by column chromatography on silica gel by eluting with hexanes-acetone.[6] Alternatively, **3b** may be alkylated with alcohol R_3OH using Mitsunobu conditions.[7] Compound **4b** can be reduced to the cyclohexyl analog **6** by catalytic hydrogenation over 10% ruthenium on carbon in ethanol at 1000 psi and 100 °C. Compound **4a** can be alkylated by stirring in THF with sodium hydride and alkyl bromide R_2Br to give **7** or **8**.

Our initial attempt to synthesize **2a** made use of standard methods.[8] Amine **4b** (R_3=*n*-$C_{12}H_{25}$) was allowed to react with 2,4,6-trimethoxyphenyl isocyanate in the presence of triethylamine in refluxing ethyl acetate, but no urea could be isolated. Urea **2a** was obtained in 18% yield when the amine was first allowed to react with *n*-butyl lithium,[9] but the low yield lead us to investigate an alternate coupling method.

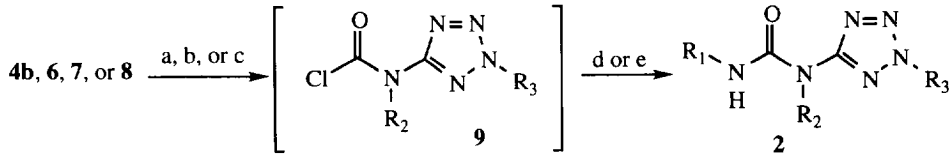
We reasoned that the coupling would be improved if the amine or the corresponding lithium amide was allowed to react with an electrophile that was more reactive than an isocyanate, such as phosgene or triphosgene. We chose to use triphosgene because it is a safer and easier to handle phosgene equivalent.[10] We envisioned a two-step, one-pot synthesis that proceeded through the carbamoyl chloride **9** as shown in Scheme 2.

Scheme 1. Synthesis of Secondary amines



(a) R_3Br, Et_3N, CH_3CN, reflux; separate by chromatography; (b) $H_{2\,(g)}$, 10% Ru/C, 1000 psi, 100 °C, EtOH; (c) BnBr, NaH, THF, 25 °C; (d) *n*-$C_7H_{15}Br$, NaH, THF, 25 °C

Scheme 2. Synthesis of Ureas 2



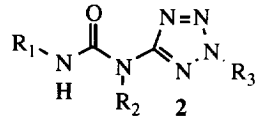
(a) i. *n*-BuLi, THF, -78 °C, 1 h; ii. 0.33$(Cl_3CO)_2CO$; (b) NaH, THF, 25 °C, 0.33$(Cl_3CO)_2CO$; (c) NaH, PhMe, 25-95 °C, 0.33$(Cl_3CO)_2CO$; (d) R_1NH_2, 2 Et_3N or 2 iPr_2NEt, 25 °C-reflux; (e) R_1NH_2, 2 NaH, THF, 25 °C

In Scheme 2, we added a solution of *n*-butyl lithium in hexanes to a stirred solution of amine **4b** (R_3=*n*-$C_{12}H_{25}$) in THF at -78 °C under nitrogen, stirred for 1 h, added 0.33 equiv of triphosgene, and warmed to room temperature. After 48 h, 2,4,6-trimethoxyaniline and 2 equiv of triethylamine were added, and the mixture was stirred to give the urea **2a** in 65% yield after purification by column chromatography.

Variations of this protocol were explored in the course of preparing additional ureas **2b-k**, and the results are shown in Table 1. Excess sodium hydride in THF at room temperature or in toluene at temperatures up to 95 °C can also be used successfully (Compounds **2b g, h, j**). The carbamoyl chlorides **9**, which presumably

are intermediates in the reaction, are stable at temperatures up to 95 °C. The acid sensitive *O*-THP group was stable in the presence of triphosgene and the carbamoyl chloride intermediates, even at elevated temperatures (**2e** and **2k**).

Table 1. Urea synthesis and in vitro ACAT inhibition and in vivo cholesterol lowering activity



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Cpd 2	R_1	R_2	R_3	Method[a]	Yield (%)	LAI IC_{50} (μM)[b,c]	APCC (%ΔTC)[d,c]
a	2,4,6-$(MeO)_3$-Ph	Ph	n-$C_{12}H_{25}$	a, d	65	0.061	-74*
b	2,4,6-$(MeO)_3$-Ph	C_6H_{11}	n-$C_{12}H_{25}$	b, d	63[e]	0.057	-67*
c	2,4,6-$(MeO)_3$-Ph	Ph	n-C_5H_{11}	a, d	60[e]	0.45	-43
d	2,4,6-$(MeO)_3$-Ph	Ph	n-$C_{14}H_{29}$	a, d	33[e,f]	0.071	-22
e	2,4,6-$(MeO)_3$-Ph	Ph	$(CH_2)_8$OTHP	a, d	59[e]	0.041	-50*
f	2,4,6-$(MeO)_3$-Ph	Ph	i-$C_{12}H_{25}$	a, e	31	0.040	NT[f]
g	2,4,6-$(MeO)_3$-Ph	Bn	n-$C_{12}H_{25}$	c, d	74[h]	0.035	NT[f]
h	2,4,6-$(MeO)_3$-Ph	n-C_7H_{15}	n-$C_{12}H_{25}$	c, d	58[e]	0.29	-20
i	2,6-$(iPr)_2$-Ph	Ph	n-$C_{12}H_{25}$	a, d	78[e]	0.047	-52*
j	2,6-$(iPr)_2$-Ph	C_6H_{11}	n-$C_{12}H_{25}$	b, d	58[e]	0.031	-10
k	2,6-$(iPr)_2$-Ph	Ph	$(CH_2)_8$OTHP	a, d	54[e]	0.016	-42

(a) See Scheme 2 for conditions; (b) Measures inhibitory activity against hepatic ACAT; (c) See Biological Methods for protocol; (d) Measures effects on plasma cholesterol levels; (e) T_{max} = 56 °C; (f) 89% based on recovered starting material; (g) NT = not tested; (h) T_{max} = 95 °C; * denotes statistical significance ($p < 0.0001$)

Biological Methods: Liver ACAT inhibitory activity was determined in vitro using [1-^{14}C]oleoyl-CoA and microsomes isolated from the livers of cholesterol-fed rats (LAI), and the results expressed as the micromolar concentration required to inhibit activity by 50% (IC_{50}).[11] Acute hypocholesterolemic activity was determined in rats by giving a single 30 mg/kg dose, by gavage, of compound suspended in carboxymethyl-cellulose (CMC)/Tween-20 in water, followed by a single high-fat, high cholesterol meal (APCC). Total serum cholesterol levels were measured the next day and the data expressed as a percent change relative to controls.[11] The ability of compound to penetrate the cell wall and inhibit macrophage ACAT was determined in cell culture using murine IC-21 macrophages (MAI), and the results expressed as an IC_{50}.[11] Systemic bioactivity was determined by testing plasma, drawn four hours post dose from a cholesterol-fed rabbit, with rat liver microsomes (ABIO). Adrenal toxicity was evaluated by an in vivo guinea pig (IVGP) screen.[12] The long-term effects on atherosclerosis were studied in an injured cholesterol-fed rabbit (ICFR) model.[13] A lesion was induced in the thoracic aortic arch by a high cholesterol diet, and drug effectiveness compared to a progression

control is determined by measuring lesion size. Another lesion was induced by injury in the iliac-femoral artery, and drug effectiveness compared to a regression control was determined by measuring both lesion and macrophage size.

Results and Discussion: The results of screening for hepatic ACAT inhibition and cholesterol lowering ability are presented in Table 1. The ureas **2** were found to be potent inhibitors, with IC_{50}'s between 0.016 and 0.45 μM. The 2,4,6-trimethoxyphenyl and 2,6-diisopropylphenyl groups were selected for R_1 as they were previously found to be optimal to obtain both in vitro and in vivo activity.[14] The *n*-dodecyl group was found to be optimal for R_3 in order to obtain efficacious cholesterol lowering in vivo, in agreement with previous observations.[5] There is no clear preference for R_2, although *n*-heptyl (**2h**) was detrimental to both in vitro and in vivo activity compared to phenyl and cyclohexyl (**2a** and **2b**). Of the potent inhibitors, **2a**, **b**, **e**, and **i** were found to most effectively lower total serum cholesterol in the acute rat model.

Theoretically, ACAT inhibitors can exhibit anti-atherosclerotic activity indirectly by lowering total serum cholesterol via inhibition of intestinal or liver ACAT, or directly by inhibiting ACAT in the arterial wall, with or without affecting total serum cholesterol levels.[2a] It is important to determine whether an ACAT inhibitor is systemically active against ACAT in the arterial wall. Therefore, compounds in Table 1 were tested for activity in the MAI and ABIO assays. The results for compounds which showed either good cholesterol lowering in APCC (>50%) or potency in MAI are listed in Table 2 along with those for CI-976.

All of the ureas listed in Table 2 were found to be bioactive at a dose of 25 mg/kg in ABIO, indicating a bioactive component is systemically available in the rabbit at that dose. Urea **2b** was also tested at a dose of 5 mg/kg and was inactive. In the MAI, **2i** was found to be more active than CI-976, while compounds **2a** and **2j** were found to be less active. Compound **2b** was found to be inactive in MAI at 0.1 μM, as were all of the other ureas in Table 1. Note that activity in the MAI depends upon two factors, the ability to penetrate the macrophage cell wall and/or to inhibit macrophage ACAT.

Thus, **2b** and **2i** were candidates for screening in our long-term, rabbit model of atherosclerosis. Both were found to be active inhibitors of liver ACAT, efficacious in vivo lowering total cholesterol, and bioactive. Conversely, **2b** and **2i** possessed different activities in MAI. Therefore, we thought they would allow us to investigate the relative importance of the direct and indirect effects of an ACAT inhibitor on slowing the progression of atherosclerotic lesion development in our long-term, rabbit model of atherosclerosis (ICFR).

First, compounds **2b** and **2i** were tested for toxicity in the guinea pig model (IVGP). Drug-related changes were not observed in livers of guinea pigs given **2b** or **2i** or in adrenals of guinea pigs given **2b**. Equivocal structural changes consisting of a slight increase of coarse vacuolation of the zona fasciculata cortical cells occurred in a minority of animals given **2i**. Therefore **2i** was not tested further.

Instead, we compared **2b** at 25 and 5 mg/kg with CI-976 at 25 mg/kg in the ICFR. CI-976 has been shown to possess anti-atherosclerotic activity in the rabbit at this dose.[13] The results are included in Table 2. Unlike CI-976, **2b** effected no statistically significant change in lesion coverage versus progression control at either dose. Urea **2b** elevated plasma triglycerides at 25 mg/kg but not at 5 mg/kg. Since elevated plasma

triglyceride levels can promote atherosclerosis, no conclusions can be made about the effect of **2b** on lesion coverage at the higher dose. However, **2b** at 5 mg/kg and CI-976 at 25 mg/kg had comparable effects on cholesterol levels, lowering total serum cholesterol 22% and 24%, respectively, versus progression control. The cholesterol lowering may be due to inhibition of intestinal and/or hepatic ACAT. Since **2b** has previously been shown to be inactive in both the bioassay at 5 mg/kg and the macrophage ACAT assay, these results suggest that CI-976 effects reduction of lesion coverage directly by inhibiting ACAT in the arterial wall, rather than indirectly by lowering total serum cholesterol levels. Based on these results, **2b** was not investigated further.

Table 2. Bioavailability and anti-atherosclerosis effects

Compound[a]	MAI IC_{50} (μM)	ABIO[b]	ICFR Thoracic Aortic Lesion Coverage (%)[c]	ICFR %ΔTC[d]	ICFR %ΔTG[e]
2a[22]	0.89	58	NT[f]	NT	NT
2b[25]	NA[g] @ 0.1 μM	58	44	-21*	+68*
2b[5]		0	49	-22*	-4
2i[22]	0.24	79	NT	NT	NT
2j[24]	3.1	77	NT	NT	NT
CI-976[25]	0.40	73	39**	-24*	-7
Progression Control	-	-	57	-	-
Regression Control	-	-	34**	-26*	-25*

(a) superscript indicates dose (mg/kg) of compound used in the ABIO and ICFR screens; (b) Expressed as percent inhibition; (c) Expressed as percent lesion coverage; (d) Percent change in total serum cholesterol relative to progression control; (e) Percent change in plasma triglycerides relative to progression control; (f) NT = not tested; (g) NA = not active; * denotes statistical significance ($p < 0.10$); ** denotes statistical significance ($p < 0.05$)

In conclusion, we have identified a novel series of tetrazole-substituted ureas **2** as ACAT inhibitors. We have also reported on a novel method for preparing ureas from weakly nucleophilic, secondary amines via intermediate carbamoyl chlorides **9**. The ureas were found to potently inhibit liver ACAT and lower total serum cholesterol in the rat. Urea **2i** was found to be more active in the macrophage ACAT assay than CI-976, a compound that has been shown to have antiatherosclerotic properties. Unfortunately, urea **2i** exhibited potential for adrenotoxicity in the guinea pig. Urea **2b** did not inhibit macrophage ACAT, but it was found to be non-toxic and efficacious in lowering total serum cholesterol. Urea **2b** and CI-976 were examined in a long-term, rabbit model of atherosclerosis to compare the relative importance of inhibition of arterial ACAT versus lowering total plasma cholesterol as direct and indirect ways, respectively, of affecting lesion size. In this model, **2b** at a dose of 5 mg/kg was shown to be equally efficacious at lowering total serum cholesterol as CI-976, but it had no affect on lesion size. This result indicates that for ACAT inhibitors to effect lesion reduction in models of atherosclerosis, they must be capable of inhibiting ACAT in the arterial wall.

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