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Modified 3-Alkyl-1,8-dibenzylxanthines as GTP-Competitive Inhibitors of Phosphoenolpyruvate Carboxykinase

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Abstract—The first non-substrate like inhibitors of human cytosolic phosphoenolpyruvate carboxykinase (PEPCK) competitive with GTP are reported. An effort to discover orally active compounds that improve glucose homeostasis in Type 2 diabetics by reversibly inhibiting PEPCK led to the discovery of 1-allyl-3-butyl-8-methylxanthine (**5**). We now report modifications at *N*-1 and *C*-8 that improved the in vitro activity of the initial xanthine HTS hit by 100-fold and a developing SAR for this class of inhibitor.
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The control of glucose production is one of the key aspects of an anti-diabetic therapy. Increased hepatic gluconeogenesis is thought to lead to fasting hyperglycemia in patients with Type 2 diabetes. The cytosolic phosphoenolpyruvate carboxykinase (PEPCK) enzyme is known to catalyze the rate-limiting step in gluconeogenesis.[1] In addition, it is reported that over-expression of PEPCK in transgenic animals results in fasting hyperglycemia and impaired glucose tolerance.[2–4] Therefore, reducing the activity of PEPCK might be expected to lead to a lowering of fasting blood glucose levels in diabetic patients. The step in glucose synthesis catalyzed by PEPCK is shown below:

Oxaloacetic acid (OAA)+

GTP$\rightleftharpoons$phosphoenolpyruvate (PEP) + GDP + CO_2

Apart from substrate analogues the only reported reversible inhibitor of PEPCK is 3-mercaptopicolinic acid (3-MPA). In the enzyme assay used in our studies 3-MPA was found to have an IC_{50} in the 20 μM range. In addition, 3-MPA has been reported to be non-competitive with respect to all substrates.[5]

We now describe modifications of the high throughput screen (HTS) hit, 1-allyl-3-butyl-8-methylxanthine (**5**), at *N*-1 and *C*-8 that improve the in vitro activity by 100-fold. The trisubstituted xanthines reported here are novel xanthines and represent the first inhibitors of human cytosolic PEPCK to compete with the natural GTP substrate. We present a SAR for *N*-1, a developing SAR for *C*-8 and a suggestion that xanthine's *N*-7 hydrogen might be required for activity.

In a search for novel inhibitors of PEPCK a HTS was initiated and hits confirmed and the effect of the compounds on PEPCK's enzymatic activity determined using recombinant human cytosolic PEPCK, expressed and purified from *Escherichia coli* as a GST-fusion protein. GTP and manganese dependent PEPCK enzyme activity catalyzes the decarboxylation of oxaloacetic acid yielding GDP and phosphoenolpyruvate (PEP). This reaction was coupled to pyruvate kinase and lactate dehydrogenase catalyzed reactions and the overall reaction rate was determined by measuring the difference in the rate of change in absorbance at 340 nM during a 20-min incubation period. In brief, 2.5 μg of recombinant human cytosolic GST-PEPCK was added to a room temperature reaction mixture which contained 0.3 mM GTP, 0.3 mM OAA, 3 mM $MgCl_2$, 0.075 mM $MnCl_2$, 30 mM potassium phosphates, pH 7.6, 1 mM dithiothreitol (DTT), 0.2 mM ADP, 1 mM NADH, 0.9 units/mL each of pyruvate kinase and lactate dehydrogenase and 1 mg/mL BSA. Compounds were dissolved in DMSO, and then added to the above mixture such that the final concentration of DMSO was

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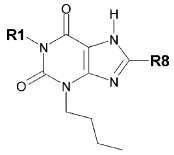
10%. DMSO at 10% had no effect on the enzyme in this assay. Individual compounds were run as duplicates per assay and the results reported as the average of the two runs. The results given with the standard deviation were of compounds assayed more than once. Doses were chosen such that each incubation included a range of eight data points for the IC_{50} determination. Assays of new compounds always included the most active compound(s) prepared up to that point to give an immediate determination as to whether a specific modification improved activity. As the activity of compounds improved the highest concentration tested was decreased, which allowed for more data points around the IC_{50} value. The result of lowering the maximum concentration was that IC_{50} values were not always obtained for less potent compounds. For these compounds, as seen in Table 1, the % inhibition at the highest concentration tested is reported.

Preparation of the majority of the xanthines reported here employed the route shown in Scheme 1 and used methods described by others working in the uracil and xanthine fields.[6–8] The 6-amino-1-butyl uracil (**1**)[6] was converted into the required 1,3-disubstituted-5,6-diamino uracils (**3a–f**) using the method described by Müller et al.[7] Acylation with the appropriate carboxylic acid[9] used the procedure of Jacobson et al.[8] to afford the acylamino uracils **4a–i**. Cyclization of the acylamino uracils **4a–i** to the 1,3,8-trisubstituted xanthines employed 10% NaOH in a mixture of methanol-water heated in a 50 °C oil bath. These conditions minimized cleavage of the *N*-acetyl bond in compounds **4b,c,f–i**, which was problematic at higher reaction temperatures. The lability of the *N*-trifluoroacetyl group in **4e** to the cyclization conditions was used to advantage in the preparation of the *C*-8 4-aminobenzyl analogue **13**.

Table 1. IC_{50} values from the enzyme assay for modifications of compound **5** at *N*-1 and *C*-8[a]

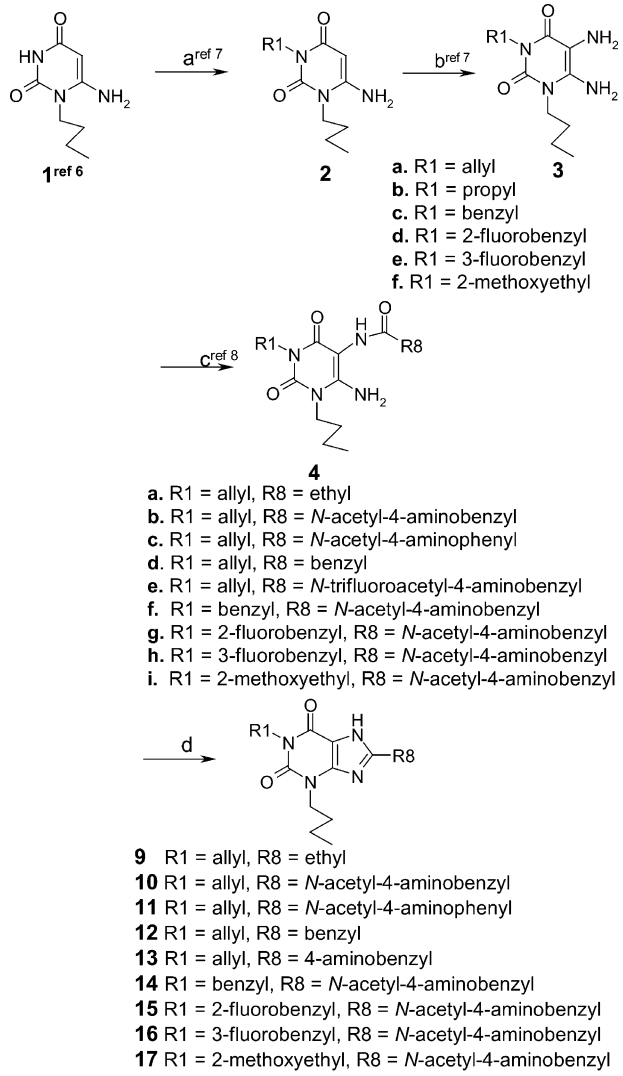


Compd	R1	R8	IC_{50}, μM[b]
5	Allyl	Methyl	225±25
8	Allyl	H	39% @ 500[c]
9	Allyl	Ethyl	100
10	Allyl	*N*-Acetyl-4-aminobenzyl	18.8±6.2(4)
11	Allyl	*N*-Acetyl-4-aminophenyl	1.7% @ 500[c]
12	Allyl	Benzyl	29% @ 25[c]
13	Allyl	4-Aminobenzyl	37
14	Benzyl	*N*-Acetyl-4-aminobenzyl	8.15±1.65(12)
15	2-Fluorobenzyl	*N*-Acetyl-4-aminobenzyl	2.11±0.60(32)
16	3-Fluorobenzyl	*N*-Acetyl-4-aminobenzyl	46% @ 25[c]
17	2-Methoxyethyl	*N*-Acetyl-4-aminobenzyl	36% @ 25[c]

[a]^{1}H NMR and MS data for compounds **8–17** are provided in ref 18.
[b]IC_{50} inhibitory values from the enzyme assay. Results showing standard deviation (sd) values were assayed more than once. If multiple assays were preformed, the number of repetitions is shown in parentheses. A single number without sd indicates a single assay with the average of the duplicate runs reported.
[c]Percent inhibition at the μM concentration indicated.

Preparation of the *C*-8 unsubstituted analogue **8** and compound **6** involved refluxing **3a** with triethylorthoformate and **3b** with triethylorthoacetate, respectively.[10]

The initial xanthine HTS lead, compound **5**, was a competitive inhibitor with respect to the substrate GTP but was a weak inhibitor of PEPCK with an IC_{50} of 225±25 μM in the forward reaction. Compounds with this level of inhibition would not normally have been pursued, however, xanthine **5** represented a drug-like hit and a structure with a number of modifiable sites. In addition compounds with a short half-life were desirable and xanthines appeared to fit that requirement; for example in humans 1,3-dimethylxanthine (theophylline), a bronchodilator, has a half-life of ~9 h, bioavailability of 96%, and the 1,3,7-trimethylxanthine (caffeine) has a half-life ~5 h, is 100% bioavailable, and both compounds are metabolized in the liver. Also later work[11] suggested that the IC_{50} values reported here might be as much as 9-fold higher than the true binding

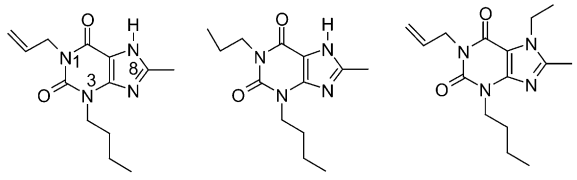


Scheme 1. Route to 3-butyl-1,8-disubstituted xanthines. Reagents and conditions: (a) (1) HMDS, $(NH_4)_2SO_4$, heat, (2) 1 equiv RCH_2X, reflux; (b) (1) $NaNO_2$, HOAc/H_2O; (2) $Na_2S_2O_4$, NH_4OH at 85–95 °C; (c) 1 equiv EDCI, imidazole (cat); DMAP (cat), DMF with the required carboxylic acid; (d) NaOH in MeOH/H_2O in a 50 °C oil bath.

constants (K_i values) due to the fact that the PEPCK assay used a saturating concentration of GTP.[12]

Assay of several xanthines from the Roche Repository in follow-up (data not shown) suggested that the π-system of the allyl group at *N*-1 was important and also that a *C*-8 benzyl group might improve activity.

Additional support for the need for a π-system at *N*-1 came from the decrease in activity of the *N*-1 saturated analogue **6**, see below. The importance of the *N*-7 hydrogen in xanthine binding was suggested by the loss of activity shown by the *N*-7 ethyl analogue **7**.[13]



5 IC_{50} 225±25μM

6 IC_{50} 375 μM

7 IC_{50} 34% @500 μM

The requirement for *C*-8 substitution was confirmed by preparation and assay of several *C*-8 modifications of compound **5** (see Table 1). The *C*-8 unsubstituted analogue **8** was >2-fold less active than the *C*-8 methyl analogue **5**. The *C*-8 ethyl analogue **9** improved activity ~2-fold over the *C*-8 methyl of the initial hit. Incorporation of the *N*-acetyl-4-aminobenzyl unit at *C*-8 giving 1-allyl-3-butyl-8-(*N*-acetyl-4-aminobenzyl)-xanthine (**10**) resulted in a >10-fold improvement in activity.

The importance of at least a one-carbon linker between the xanthine and the phenyl ring at *C*-8 was demonstrated by the loss of activity observed with the *N*-acetyl-4-aminophenyl analogue **11**. Although the *C*-8 phenyl analogue **11** showed only 1.7% inhibition at 500 μM in the PEPCK assay, other 1,3,8-trisubstituted xanthines with a phenyl group at *C*-8 have been reported to be adenosine receptor (AR) antagonists.[14] While the compounds described here have not been assayed against ARs, a reported comparison of a 1,3-dimethyl-8 benzylxanthine with its *C*-8 phenyl-analogue in an A_{2B}AR assay showed that the *C*-8 benzyl compound was >74-fold weaker than the corresponding phenyl analogue.[15] This, and the size of the *N*-1, *N*-3 groups, suggested that the compounds described here should be weak AR inhibitors at best.

Additional modifications at *C*-8 explored in this compound class, see Table 1, included the unsubstituted benzyl analogue **12** and the 4-aminobenzyl analogue **13** both of which retained activity but were slightly less active when compared to the *N*-acetyl-4-aminobenzyl analogue **10**. These results suggested that substitution on the phenyl ring of the *C*-8 benzyl group might be important. Further modifications at *C*-8 will be described in future papers.

The need for a π-system at *N*-1, suggested by compound **6**, led to the preparation of the *N*-1 benzyl analogue **14**, see Table 1, which gave a >20-fold improvement over the activity of the original hit **5**. Modeling based on the X-ray structure of xanthine analogue **10** bound to PEPCK[11] suggested that a H-bond from PEPCK's Asn533 side chain amide to an *ortho*-substituent on the phenyl ring of the *N*-1 benzyl unit was possible. The 2-fluorobenzyl analogue **15** was prepared and its assay indicated that incorporation of the 2-fluorobenzyl moiety improved the activity nearly 4-fold over that of the corresponding *N*-1 benzyl analogue **14**.

The choice of the fluoro group was based on its small size and excellent ability to accept H-bonds (van der Waals' radii in Å, F 1.47; H 1.20; O 1.52[16] and electronegativity F 4.0 vs O 3.65[17]). This subsequently proved to have been a critical choice, as neither the 2-hydroxy nor a 2-fluorobenzyl with other groups on the phenyl ring (S. Pietranico, W. Yun, Roche Research Center, personal communication) were as active as the 2-fluorobenzyl analogue **15**. These and other results suggested the presence of a small *N*-1 binding pocket.

The possibility that the improved activity of **15** resulted from the 2-fluoro-group decreasing the electron density of the *N*-1 phenyl ring was explored by preparation of the identical compound with the fluorine at the 3-position. Once again the small size of fluorine allowed the examination of this possibility. The *N*-1 3-fluorobenzyl analogue **16**, as shown in Table 1, was >10-fold less active relative to the 2-fluorobenzyl analogue **15** and thus provided no support for this hypothesis. The decreased activity of the *N*-1 methoxyethyl analogue **17**, which contains only a H-bond acceptor, when compared to either the benzyl or 2-fluorobenzyl analogues **14** and **15**, respectively, provided additional support for the importance of a π-system at *N*-1 for enhanced binding.

In summary we describe here the first GTP-competitive inhibitors of human cytosolic PEPCK and modifications to *N*-1 and *C*-8 that improved the activity of the initial HTS hit by >100-fold. Assay data is presented that suggests the *N*-7 hydrogen, a π-system at *N*-1 and a benzyl unit at *C*-8 are important for inhibitory activity. The optimal modification found for *N*-1 was the 2-fluorobenzyl group.

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12. For a competitive inhibitor the K_i will be lower than the IC_{50} by the factor $[1 + S/K_M]$, where S is the concentration of GTP in the assay, and K_M is the Michaelis constant of the enzyme for GTP. In simple terms, the higher the concentration of GTP in the assay, the higher the measured IC_{50} for a competitive inhibitor.
13. The *N*-7 ethyl analogue **7** was obtained as a side product when **5** was reprepared from the reaction of the diaminouracil **3a** with triethylorthoacetate.
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18. **Spectra general information**: ^{1}H NMR spectra were acquired with a Varian Mercury NMR spectrometer at 300 MHz. The samples were dissolved in either $CDCl_3$ or DMSO-d_6 as noted. The chemical shifts are referenced to $CHCl_3$ at 7.26 ppm or DMSO-d_5 at 2.50 ppm; exchangeable protons are denoted by ex. High resolution mass spectra were obtained on a Micromass AutoSpec instrument. The low resolution mass spectra were run as APCi on a Micromass Platform II instrument.

1-Allyl-3-butyl-3,7-dihydropurine-2,6-dione (**8**): ^{1}H NMR (DMSO-d_6) δ 0.90 (t, 3H), 1.30 (m, 2H), 1.65 (m, 2H), 4.00 (t, 2H), 4.48 (br d, 2H), 5.06 (m, 2H), 5.85 (m, 1H), 8.06 (s, 1H ex), 13.6 (s, 1H ex); HRMS calcd for $C_{12}H_{16}N_4O_2$ 248.1273; Observed 248.1274.

1-Allyl-3-butyl-8-ethyl-3,7-dihydropurine-2,6-dione (**9**): ^{1}H NMR ($CHCl_3$) δ 0.96 (t, 3H), 1.40 (t overlapping m, 5H), 1.77 (m, 2H), 2.88 (q, 2H), 4.13 (t, 2H), 4.68 (br d, 2H), 5.20 (m, 2H), 5.94 (m, 1H), 11.87 (br s, 1H ex); LRMS calcd for $C_{14}H_{20}N_4O_2$ 276; Observed M + H 277.

N-[4-(1-Allyl-3-butyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-8-ylmethyl)-phenyl]-acetamide (**10**): ^{1}H NMR (DMSO-d_6) δ 0.87 (t, 3H), 1.26 (m, 2H), 1.61 (m, 2H), 2.00 (s, 3H), 3.94 (t, 2H), 3.97 (s, 2H), 4.44 (br d, 2H), 5.06 (m, 2H), 5.82 (m, 1H), 7.17 (d, 2H), 7.47 (d, 2H), 9.89 (s, 1H ex), 13.4 (s, 1H ex); HRMS calcd for $C_{21}H_{25}N_5O_3$ 395.1957; Observed 395.1947.

1-Allyl-3-butyl-8-(*N*-acetyl-4-aminophenyl)-3,7-dihydropurine-2,6-dione (**11**): ^{1}H NMR (DMSO-d_6) δ 0.93 (t, 3H), 1.33 (m, 2H), 1.71 (m, 2H), 2.08 (s, 3H), 4.06 (t, 2H), 4.51 (br d, 2H), 5.08 (m, 2H), 5.87 (m, 1H), 7.81 (d, 2H), 8.06 (d, 2H), 10.2 (s, 1H ex), 13.69 (s, 1H ex); LRMS calcd for $C_{20}H_{23}N_5O_3$ 381; Observed M + H 382.

1-Allyl-8-benzyl-3-butyl-3,7-dihydro-purine-2,6-dione (**12**): ^{1}H NMR (DMSO-d_6) δ 0.87 (t, 3H), 1.26 (m, 2H), 1.61 (m, 2H), 3.94 (t, 2H), 4.03 (s, 2H), 4.44 (d, 2H), 5.04 (m, 2H), 5.80 (m, 1H), 7.23 (m, 5H), 13.42 (s, 1H ex); HRMS calcd for $C_{19}H_{22}N_4O_2$ M + 338.1742; Observed 338.1742.

1-Allyl-8-(4-aminobenzyl)-3-butyl-3,7-dihydropurine-2,6-dione (**13**): ^{1}H NMR (DMSO-d_6–D_2O) δ 0.87 (t, 3H), 1.26 (m, 2H), 1.61 (m, 2H), 3.82 (s, 2H), 3.92 (t, 2H), 4.44 (d, 2H), 5.00 (m, 2H), 5.80 (m, 1H), 6.44 (d, 2H), 6.90 (d, 2H); HRMS calcd for $C_{19}H_{23}N_5O_2$ 354.1931; Observed 354.1928.

N-[4-(1-benzyl-3-butyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-8-ylmethyl)-phenyl]-acetamide (**14**): ^{1}H NMR (DMSO-d_6) δ 0.86 (t, 3H); 1.26 (m, 2H); 1.61 (m, 2H); 2.00 (s, 3H), 3.94 (t, 2H), 3.98 (s, 2H), 5.04 (s, 2H), 7.17–7.30 (m, 7H), 7.47 (d, 2H), 9.89 (s, 1H ex), 13.43 (s, 1H ex); HRMS calcd for $C_{25}H_{27}N_5O_3$ 446.2192; Observed 446.2199.

N-{4-[1-(2-Fluoro-benzyl)-3-butyl-2,6-dioxo-2,3,6,7-tetra-hydro-1*H*-purin-8-ylmethyl]phenyl}-acetamide (**15**): ^{1}H NMR (DMSO-d_6) δ 0.86 (t, 3H), 1.26 (m, 2H), 1.60 (m, 2H), 2.00 (s, 3H), 3.95 (t, 2H), 3.99 (s, 2H), 5.09 (s, 2H), 6.97–7.27 (m, 6H), 7.48 (d, 2H), 9.82 (s, 1H ex), 13.45 (br s, 1H ex); HRMS calcd for $C_{25}H_{26}N_5O_3F$ M + 464.2098; Observed 464.2086.

N-{4-[3-Butyl-1-(3-fluorobenzyl)-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-8-ylmethyl]-phenyl}-acetamide (**16**): ^{1}H NMR (DMSO-d_6) δ 0.94 (t, 3H), 1.34 (m, 2H), 1.70 (m, 2H), 2.08 (s, 3H), 4.03 (t, 2H), 4.06 (s, 2H), 5.13 (s, 2H), 7.10–7.60 (m, 8H), 9.97 (s, 1H ex), 13.52 (s, 1H ex); HRMS calcd for $C_{25}H_{26}N_5O_3F$ 463.2020; Observed 463.2018.

1-(2-Methoxyethyl)-3-butyl-8-(*N*-acetyl-4-aminobenzyl)-3,7-dihydropurine-2,6-dione (**17**): ^{1}H NMR (DMSO-d_6) δ 0.89 (t, 3H), 1.28 (m, 2H), 1.62 (m, 2H), 2.01 (s, 3H), 3.32 (s, 3H), 3.47 (t, 2H), 3.95 (t, 2H), 3.98 (s, 2H), 4.05 (t, 2H), 7.18 (d, 2H), 7.49 (d, 2H), 9.91 (s, 1H ex), 13.40 (s, 1H ex); HRMS calcd for $C_{21}H_{27}N_5O_4$ 413.2063; Observed 413.2064.