Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2795–2798

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of *Escherichia coli* Glucosamine Synthetase by Novel Electrophilic Analogues of Glutamine—Comparison with 6-Diazo-5-oxo-norleucine

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Received 4 August 2000; accepted 3 October 2000

Abstract—A series of electrophilic glutamine analogues based on 6-diazo-5-oxo-norleucine has been prepared, using novel synthetic routes, and evaluated as inhibitors of *Escherichia coli*. glucosamine synthetase. The γ-dimethylsulphonium salt analogue of glutamine was found to be one of the most potent inactivators of this enzyme yet reported, with an apparent second order rate constant (k_2/K_i) of $3.5\times10^5\,M^{-1}\,min^{-1}$.

The maintenance of the structural integrity of the outer cell wall of bacteria and fungi is essential for their continued survival. Glucosamine synthetase (L-glutamine:D-fructose-6-phosphate amidotransferase; EC 2.6.1.16) (GS) plays a pivotal role in the biosynthesis of amino sugar-containing macromolecules that constitute the major structural outer membrane proteins of these organisms, such as the peptidoglycans in bacteria and chitin in fungi.[1,2] Compounds designed to selectively inhibit this enzyme could therefore constitute a potential source of new bactericidal and fungicidal agents.

One possible strategy applicable to the design of such agents is the fact that GS belongs to the family of amidotransferases, all of which possess an active site cysteine nucleophile that can be affinity labelled by the glutamine analogue 6-diazo-5-oxo-norleucine (DON)[3,4] (**1**). Inactivation studies with purified *Escherichia coli* GS, employing ^{14}C6-labelled DON, established that the active site cysteine residue occupied position 1 of the primary sequence of the enzyme.[5] Consequently, as part of a programme directed towards the development of new amidotransferase inhibitors with putative antimicrobial activity, we wish to report the effectiveness of a series of alternative glutamine analogues as inhibitors of glucosamine synthetase. This paper describes the synthesis and kinetic testing of a series of analogues in which the side chain amide (-$CONH_2$) of glutamine has been replaced by various electrophilic species including γ-halomethyl ketone (-$COCH_2Cl$; -$COCH_2Br$) (**2**, **3**), γ-nitrile (-C≡N) (**4**), γ-dimethylsulphonium salt (-$COCH_2S^+(Me)_2$) (**5**) and γ-diazoethyl ketone (-$COCH(CH_3)CHN_2$) (**6**) (Fig. 1).

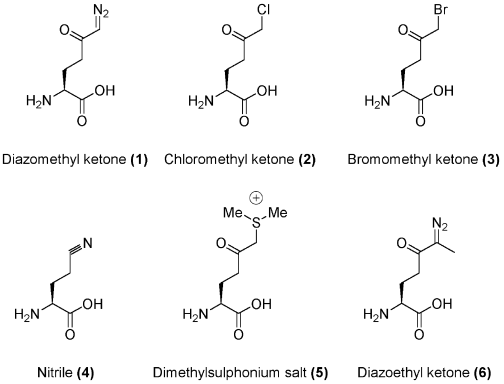

Figure 1. Electrophilic analogues of glutamine.

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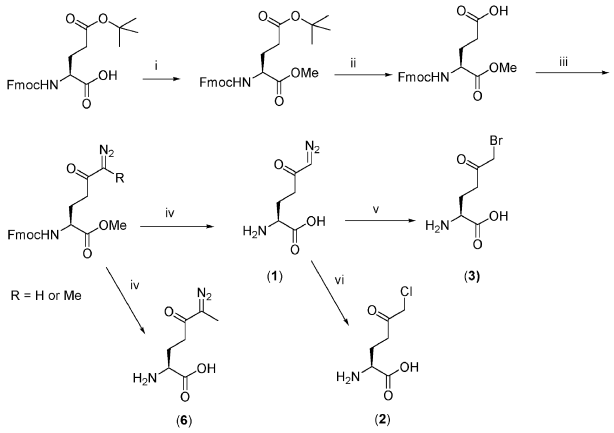
0960-894X/00/$ - see front matter
PII: S0960-894X(00)00565-5

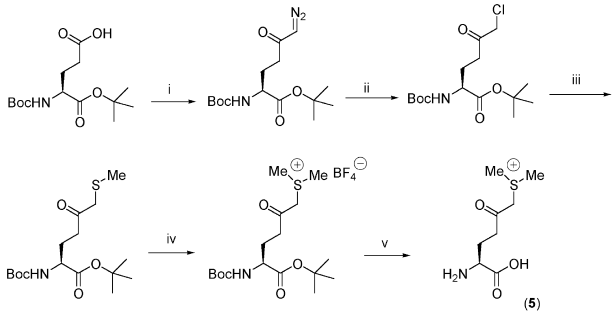
Chemistry

Schemes 1 and 2 outline the synthetic strategies employed for the synthesis of the putative inhibitors utilized in this study. Scheme 1 outlines the synthesis of 6-diazo-5-oxo-norleucine (**1**), from *N*-α-Fmoc-glutamic acid-γ-*tert*-butyl ester, and illustrates the use of this intermediate for the preparation of halomethyl ketone derivatives **2** and **3**. The γ-diazoethyl ketone analogue (**6**) was prepared via the acylation of diazoethane (generated from *N*-nitroso-β-isobutyl methyl ketone).



Scheme 1. Synthesis of DON and halomethyl ketone analogues: (i) ethereal diazomethane; (ii) 50% (v/v) TFA/DCM; (iii) *N*-methylmorpholine, isopropenyl chloroformate at 0 °C, then add ethereal diazomethane or diazoethane; (iv) 4 M NaOH; (v) 30% (v/v) HBr/AcOH; (vi) ethereal HCl.

Scheme 2 outlines the synthesis of 6-dimethylsulphonium-5-oxo-norleucine (**5**) from *N*-α-Boc-(L)-glutamic acid-α-*tert*-butyl ester. The γ-nitrile derivative (**4**) was synthesized via the dehydration of (L)-glutamine, essentially according to the method of Stüber et al.[6] Compound identity and purity was assessed by ^{1}H NMR, TLC, ESI-MS and elemental analysis.



Scheme 2. Synthesis of γ-dimethylsulphonium ketone analogue: (i) *N*-methylmorpholine, isopropenyl chloroformate at 0 °C, then add ethereal diazomethane; (ii) ethereal HCl; (iii) sodium methane thiolate; (iv) MeI/$AgBF_4$ in nitromethane; (v) dimethylsulphide/TFA.

Inactivation Studies

E. coli glucosamine synthetase was purified from *E. coli* (strain K12), according to a previously published procedure.[6]

For time-dependent inactivation assays, glucosamine synthetase (~17 μg) was pre-incubated in 100 mM potassium phosphate buffer, pH 7.5, containing 10 mM fructose-6-phosphate and 50 mM KCl, for 15 min at 37 °C. The reaction was initiated by addition of the inhibitor under investigation, and at timed intervals, 20 μL aliquots were removed and diluted 40-fold into the same buffer, containing 6 mM (L)-glutamine. Residual activity was then determined by the colorimetric assay for glutamate described by Shiio and Ishii.[7]

Inactivation, assessed using the method of Kitz and Wilson,[8] was found to be dependent on both incubation time and concentration of inhibitor used. A linear correlation was observed from a plot of the natural logarithm of residual activity against incubation time, from which the inactivation half time ($t_{1/2}$) at each inhibitor concentration could be read directly. The apparent pseudo first-order rate constant (k_{obs}) was calculated according to:

$$k_{obs}=1_n2/t_{1/2}$$

A hyperbolic curve was obtained when these constants were plotted against inhibitor concentration. This behaviour is consistent with the formation of a reversible enzyme–inhibitor complex prior to inactivation (**1**):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E.I \overset{k_{+2}}{\rightarrow} E-I \quad (1)$$

where E.I is the reversible enzyme–inhibitor complex E–I is the irreversible enzyme–inhibitor complex and k_{+2} is the limiting rate constant for inactivation. Assuming that [I] > >[E] and that the reversible complex is at all times in equilibrium with the enzyme and inhibitor, Kitz and Wilson derived the following equation:

$$\frac{1}{k_{obs}} = \frac{K_i}{k_{+2}} \times \frac{1}{[I]} + \frac{1}{k_{+2}} \quad (2)$$

where k_{obs} is the apparent pseudo first order rate constant, k_{+2} is the inactivation rate constant at saturating inhibitor concentration and K_i is the steady state inactivation constant. A plot of $1/k_{obs}$ against 1/[I] is a straight line with the ordinate intercept equal to $1/_{k+2}$ and the abcissa intercept equal to $-1/K_i$. Data obtained from these studies is summarized in Table 1.

Results and Discussion

Results clearly show that, in addition to DON (**1**), the enzyme is inactivated by a number of other electrophilic carbonyl analogues. Kinetic analysis of inhibition by the dimethylsulphonium salt (**5**), diazoethyl- (**6**) and halomethyl ketone (**2** and **3**) analogues was indicative of irreversible inhibition in each instance. One observation of particular interest was the inactivity of the γ-nitrile derivative (**4**), even at concentrations as high as 200 μM. Peptides incorporating the nitrile group have previously

been shown to act as good inhibitors of cysteine peptidases,[9] their lack of activity against glucosamine synthetase suggests that the presence of a carbonyl group at the γ position may be one important requirement for effective binding and inhibition. It is worth pointing out however, that our nitrile-based compound (**4**) has a shorter side chain (2-carbon, compared with 3-carbon, for compounds **1**–**3** and **5**–**6**) which may be an additional or alternative reason for the apparent lack of activity of this compound.

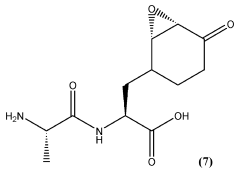
Table 1 lists the kinetic constants obtained for the inactivation of *E. coli* GS by electrophilic glutamine analogues prepared in this study.

It is clear from this table that, of the γ-halomethyl and diazomethyl ketone derivatives of glutamic acid, the bromomethyl ketone analogue (**3**) is the most potent inactivator of glucosamine synthetase, with an overall second order rate constant (k_{+2}/K_i) of 150,000 M^{-1} min^{-1}. This is some 2-fold more potent than DON (**1**) (70000 M^{-1} min^{-1}). The improved potency of the bromomethyl ketone derivative over DON can be attributed to a 3-fold higher affinity with which the former binds to the enzyme, prior to covalent modification, although this is offset somewhat by the slightly higher rate of covalent complex formation (k_{+2}), between the latter and glucosamine synthetase.

The greatly enhanced efficacy of the bromomethyl compared with the chloromethyl ketone can similarly be attributed to the enhanced affinity of the former with the enzyme (20-fold greater, reflected in the respective K_i values for both). Additionally, the 2-fold greater rate constant for the formation of the covalent complex also contributes to this greater overall efficiency.

Comparisons between the effectiveness of the diazoethyl ketone analogue (**6**) and DON illustrates that the latter is some 3.5-fold more active (20,000 M^{-1} min^{-1} compared with 70,000 M^{-1} min^{-1}). The reduced potency of the diazo*ethyl* ketone can be attributed to a very pronounced decrease in the first-order rate constant for covalent complex formation (35-fold), compared with DON. It is interesting to note, however, that affinity for the enzyme is actually higher (8-fold) with the diazoethyl analogue, thus partially offsetting the loss in reactivity. It would therefore appear that the introduction of the methyl substituent α to the departing diazo moiety enhances affinity, while reducing the electrophilicity of the methane -CH- centre, thus reducing inherent chemical reactivity of the molecule.

The most promising results appeared with the dimethylsulphonium salt. This proved to be an exceptional inhibitor of glucosamine synthetase, exhibiting a second-order rate constant, (k_{+2}/K_i) of 320,000 M^{-1} min^{-1}, some 2-fold greater than the bromomethyl ketone. The improved potency of the dimethylsulphonium salt can be attributed to the 7-fold higher affinity with which this compound binds to the enzyme prior to covalent complex formation.



(7)

The chief drawback with the use of glucosamine synthetase inhibitors as anti-microbial compounds, is their inherent toxicity. DON inactivates the mammalian form of the enzyme, and is also known to block glutaminase. One interesting approach to the utilization of glucosamine synthetase inhibitors as antibiotics, is their incorporation into peptidase-activated prodrugs. In addition to the naturally occurring tripeptides alazopeptin[10] and duazomycin,[11] which both carry intermolecular DON residues, the compound bacilysin (**7**) (tetaine), a dipeptide containing (L)-β-(2,3-epoxycyclohexan-4-one)-alanine (anticapsin),[12] which is released after the action of an unidentified intracellular aminopeptidase, is a potent anti-fungal and anti-bacterial agent. Importantly, bacilysin itself has no inherent activity against glucosamine synthetase, and as such, reduced toxicity. Thus incorporation of the dimethylsulphonium ketone into peptide prodrugs, with selective susceptibility to bacterial peptidases, may provide a means to target this potent inhibitor into bacterial and/or fungal cells.

Table 1. Steady-state inactivation rate constants (K_i), limiting rate constants for inactivation (k_{+2}) and second order rate constants (k_{+2}/K_i) for compounds **1**–**6**

Compounds	K_i[a] (μM)	k_{+2} (min^{-1})	k_{+2}/K_i (μM^{-1} min^{-1}
1	9.5 (±0.40)	0.7 (±0.04)	0.07 (±0.002)
2	62.5 (±3.7)	0.19 (±0.007)	0.003 (±0.0001)
3	2.7 (±0.1)	0.4 (±0.06)	0.15 (±0.02)
4	NI[b]	NI[b]	NI[b]
5	0.37 (±0.07)	0.12 (±0.03)	0.32 (±0.04)
6	1.1 (±0.1)	0.02 (±0.005)	0.02 (±0.007)

[a]Values are means of four determinations; standard deviation is given in parentheses.
[b]NI = no inhibition at 200 μM.

Acknowledgements

This work was supported by the award of an MRC (UK) project grant (No. 8803936) to B.W. and A.J.H.

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