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AMIDE AND ESTER DERIVATIVES OF N^3-*TRANS*-EPOXYSUCCINOYL-L-2,3-DIAMINOPROPANOIC ACID: INHIBITORS OF GLUCOSAMINE-6-PHOSPHATE SYNTHASE

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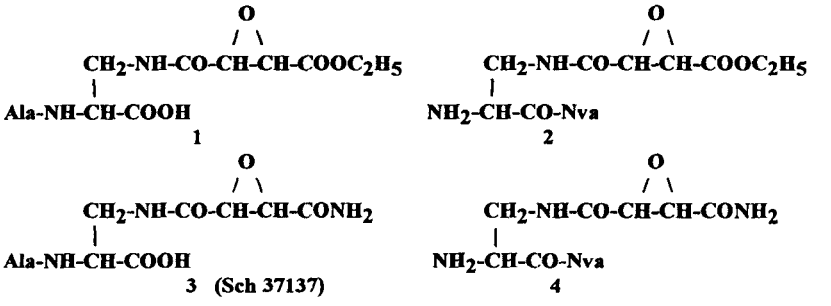
Several analogs **5**, **6**, **7**, **8**, **10** and **11** of the C-terminal fragment of a peptide antibiotic Sch 37137 were designed and tested as inhibitors of glucosamine-6-phosphate synthase from *Saccharomyces cerevisiae*. From IC_{50} values and kinetic parameters of inhibition of glucosamine-6-phosphate synthase by compounds **5–11** it has been found that the inhibitory potency of these compounds follows the order: **6** > **5** > **8** > **9** > **7**, **10**, **11**. This suggests that an inhibitor with a primary amido group binds better to the active site of the enzyme than other inhibitors. The order of reactivity of compounds **5–11** may be attributed to a steric inability of the inhibitor to fit into the active site of the enzyme and also indicates the importance of the chirality of *trans*-epoxysuccinic acid on the inhibitory properties of the synthesized compounds.

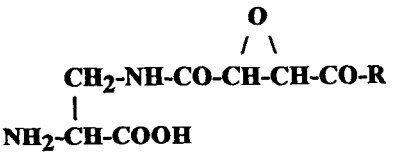
KEY WORDS: Glucosamine-6-phosphate synthase, derivatives of N^3-*trans*-epoxysuccinoyl-L-2,3-diaminopropanoic acid as enzyme inhibitors

INTRODUCTION

In recent publications,[1,2] we have reported the synthesis and anticandidal properties of analogs of a peptide antibiotic Sch 37137. Of the synthesized peptides those containing the ethyl L-*trans*-epoxysuccinate (**1**, **2**) as an acyl moiety, in contrary to compounds with an amide of DL-*trans*-epoxysuccinic acid (**3**, **4**) showed a weak anticandidal activity.



Antimicrobial properties of this type of compounds resulted from peptide transport via peptide permeases into the microbial cell[3] and subsequent enzymatic hydrolysis with the formation of an active species which may inactivate glucosamine-6-phosphate synthase,[4] an important enzyme in the biochemical formation of cell-wall macromolecules (peptidoglycan in bacteria, chitin and mannoproteins in fungi).[5] It was suggested that the terminal amino acid containing the ethyl ester residue in compounds **1** and **2** may act as a weak inactivator of that enzyme and displays a much slower rate of inactivation of glucosamine-6-phosphate synthase than corresponding compounds with an amido group. It is therefore of interest to synthesize and



5 R = NH_2

6 R = NH_2 and (2*R*,3*R*)-*trans*-epoxysuccinic acid

7 R = NH_2 and (2*S*,3*S*)-*trans*-epoxysuccinic acid

8 R = OCH_3

9 R = OC_2H_5

10 R = $NHCH_3$

11 R = $N(CH_3)_2$

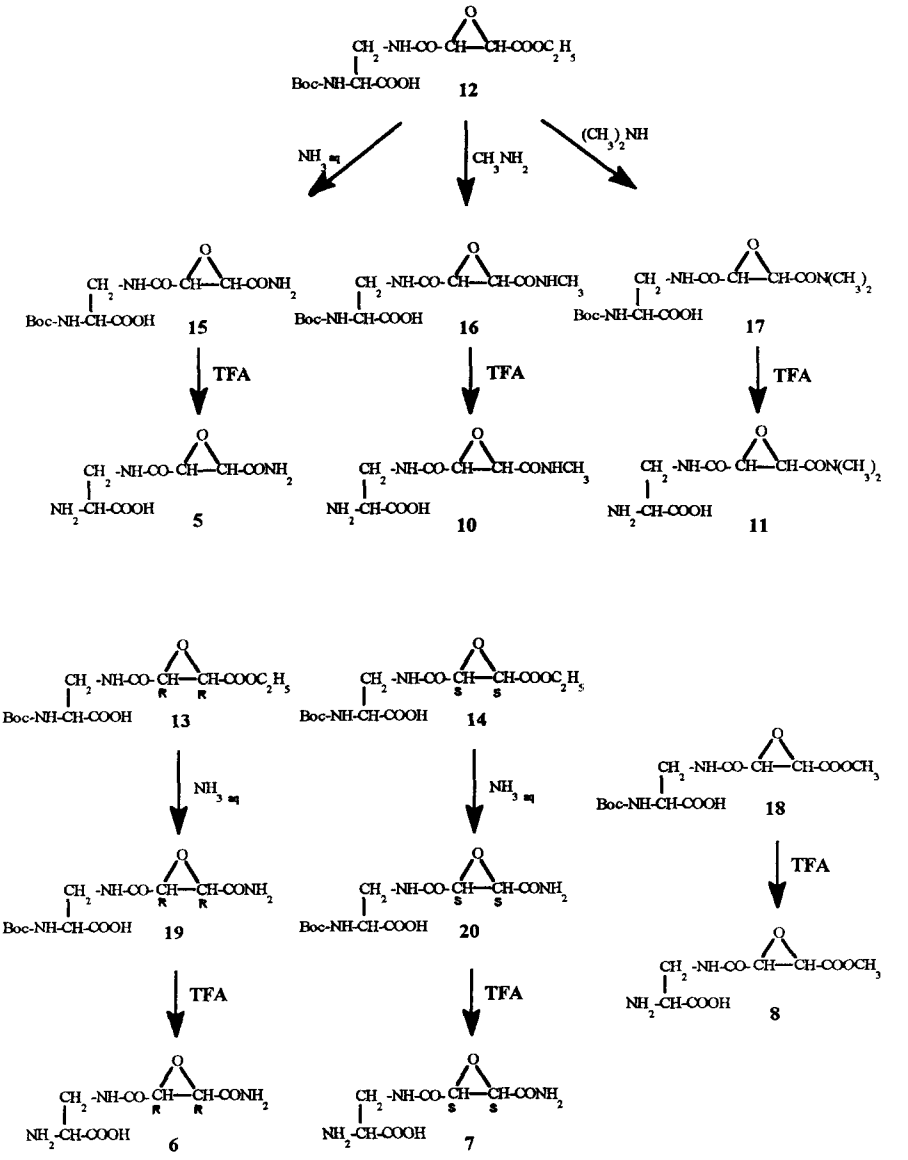
compare inhibitory properties of a series of compounds with various substituents at the carboxyl group (ester and amide derivatives). In order to determine the influence of configuration of *trans*-epoxysuccinic on the inhibitory properties of the projected inhibitors, the compounds containing (2**R**, 3**R**) and (2**R**, 3**R**)-*trans*-epoxysuccinic acid were also prepared. Moreover, these data may support a likely explanation for the mechanism of inactivation of glucosamine-6-phosphate synthase and provide more information on the inhibitor — enzyme interaction.

EXPERIMENTAL

Chemistry

N^2-tert-Butoxycarbonyl-N^3-DL-3-trans-ethoxycarbonyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **12** was prepared according to the method described earlier[1] and was used for the preparation of compounds **5**, **10** and **11**, (Scheme 1). *N^2-tert-butoxycarbonyl-N^3-D-trans-ethoxycarbonyloxirane-2-carbonyl-L-2, 3-diaminopropanoic*

acid **13** *and* N^2*-tert-butoxycarbonyl-*N^3*-L-trans-ethoxycarbonyloxirane-2-carbonyl-2,3-diaminopropanoic acid* **14** were also obtained by the previously published procedure.[2] Compound **9** in the form of its trifluoroacetate salt was also obtained following our earlier described procedure.[1]



SCHEME 1 Synthesis of compounds **5–8**, **10** and **11**.

N^2-tert-Butoxycarbonyl-N^3-DL-3-trans-carbamoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **15**. The ethyl ester **12** (692 mg, 2 mmol) was stirred with 29% cold ammonia (20 ml) for 2 h with intensive stirring. Then ammonia was evaporated, the crude residue was dissolved in a small volume of water (5 ml) and passed through a small column of Amberlite CG 50 H^+. The product was eluted with water-methanol (2:1, 50 ml), evaporated to dryness leaving an oily residue which was crystallized upon addition of ethyl ether (560 mg, 88%). Mp.72–74°C, $[\alpha]_{578} = -10.4°$C (c=1, MeOH), NMR (DMSO-d_6) δ = 1.2(9H,s), 3.4(2H,s), 3.5–3.7(2H,m), 4.1(1H,m), 6.2(1H,br.s), 6.8(1H, br.s) 7.6–7.8(2H,br.s) Anal. Calc. for $C_{12}H_{19}N_3O_7$: C, 45.42; H, 6.03; N, 13.24; Found: C, 45.21; H, 5.90; N, 13.32%.

N^2-tert-Butoxycarbonyl-N^3-DL-trans-methylcarbomoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **16**.This compound was prepared from the ethyl ester **12** and 40% aqueous methylamine as described for the preparation of **15**, as an amorphous solid (443 mg, 67%), $[\alpha]_{578} = -9.6°$, (c=1, MeOH), NMR (DMSO-d_6), δ = 1.3(9H,s), 2.8(3H,m), 3.45(2H,s), 3.6–3.7(2H,m), 4.2(1H,m), 6.3(1H,br.s), 6.9(1H,br.s), 7.5(1H,br.s). Anal. Calc. for $C_{13}H_{21}N_3O_7$: C, 47.12; H, 6.38; N, 12.68; Found: C, 47.02; H, 6.21; N, 12.53%.

N^2-tert-Butoxycarbonyl-N^3-DL-trans-dimethylcarbamoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **17**. This compound was obtained from compound **12** and 25% ethanolic dimethylamine solution as described for **15**, as an amorphous powder (380 mg, 55%), $[\alpha]_{578} = -9.8°$ (c=1, MeOH), NMR (DMSO-d_6) δ = 1.25(9H,s), 2.9(6H,m), 3.5(2H,s), 3.6–3.7(2H,m), 4.3(1H,m), 6.4(1H,br.s), 6.9(1H,br.s). Anal. Calc. for $C_{14}H_{23}N_3O_7$: C, 48.68; H, 6.71; N, 12.16; Found: C, 48.63; H, 6.58; N, 12.18%.

N^2-tert-Butoxycarbonyl-N^3-DL-trans-methoxycarbonyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **18** was prepared from N^2-tert-butoxycarbonyl-L-2,3-diaminopropanoic acid[6] and monomethyl DL-*trans*-epoxysuccinate[7] according to the procedure used for preparation of **12**. Yield 567 mg, 85%, mp. 51–52°C, $[\alpha]_{578} = -12.2°$ (c=1, MeOH), NMR (DMSO-d_6) δ = 1.3(9H,s), 3.6(2H,s), 3.7(2H,m), 3.8(3H,s), 4.1(1H,m), 6.6(1H,m), 7.1(1H,m). Anal. Calc. for $C_{13}H_{20}N_2O_8$: C, 46.98; H, 6.06; N, 8.42; Found: C, 46.67; H, 6.12; N, 8.23%.

N^2-tert-Butoxycarbonyl-N^3-DL-trans-carbamoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **19** was prepared from the ester **13** and aqueous ammonia as described for preparation of **15**. Yield 545 mg, 86%, as an amorphous powder, $[\alpha]_{578} = -44.2°$ (c = 1, MeOH). Anal. Calc. for $C_{12}H_{19}N_3O_7$: C, 45.42; H, 6.03; N,13.24; Found: C, 45.27; H, 6.12; N, 13.02%.

N^2-tert-Butoxycarbonyl-N^3-L-trans-carbamoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid **20**. This compound was obtained from the ester **14** and aqueous ammonia as described for preparation of **15**. Yield 550 mg, 87%, as an amorphous powder, $[\alpha]_{578} = +25.8°$ (c = 1, MeOH). Anal. Calc. for $C_{12}H_{19}N_3O_7$: C, 45.42; H, 6.03; N, 13.24; Found: C, 45.18; H, 5.96; N, 13.28%.

N^3-DL-trans-carbamoyloxirane-2-carbonyl-L-2,3-diaminopropanoic acid trifluroacetate **5**. Compound **15** (634 mg, 2 mmol) was dissolved in cold trifluroacetate acid (10 ml) and stored for 2 h. Excess TFA was evaporated in vacuo, the residue was triturated with ethyl ether and the precipitate was filtered off, dried in vacuo over KOH pellets. Yield 680 mg, 93%, mp. 92–94°C, $[\alpha]_{578} = -19.8°$ (c = 1, H_2O). Anal.

Calc. for $C_7H_{11}N_3O_7CF_3COOH$: C, 29.75; H, 3.32; N, 11.56; Found: C, 29.63; H, 3.22; N, 11.65%.

N^3-D-trans-carbamoyloxirane-2-carbonyl-L-2,3-diaminopropanoic acid trifluroacetate **6**. This was prepared from **19** as described for **5**. Yield 660 mg, 91%. Mp.122–125°C $[\alpha]_{578} = -44°$ (c = 1, H_2O). Anal. Calc. for $C_7H_{11}N_3O_7CF_3COOH$: C, 29.75; H, 3.32; N, 11.56; Found: C, 29.54; H, 3.23; N, 11.44%.

N^3-L-trans-carbamoyloxirane-2-carbonyl-L-2,3-diaminopropanoic acid trifluroacetate **7**. This compound was prepared from **20** as described for **5**. Yield 670 mg, 92%. Mp.134–136°C, $[\alpha]_{578} = +10.2°$ (c=1, H_2O). Anal. Calc. for $C_7H_{11}N_3O_7CF_3COOH$: C, 29.75; H, 3.32; N, 11.56; Found: C, 29.62; H, 3.15; N, 11.58%.

N^3-DL-3-trans-methylcarbamoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid trifluoroacetate 10. This compound was prepared from **16** analogously as described for **5**. Yield 360 mg, 90% as an amorphous powder, $[\alpha]_{578}$ = (c=1, MeOH). Anal.Calc. for $C_8H_{13}N_3O_7CF_3COOH$: C, 31.83; H, 3.74; N, 11.13; Found: C, 31.75; H, 3.52; N, 11.19%.

N^3-DL-3-trans-dimethylcarbamoyloxirane-2-carbonyl-L-2, 3-diaminopropanoic acid trifluoroacetate **11**. This was obtained from **17** as described for **5**. Yield 350 mg, 89% as an amorphous powder $[\alpha]_{578} = 22.8°$ (c=1, MeOH). Anal. Calc. for $C_9H_{15}N_3O_7CF_3$ COOH: C, 34.56; H, 4.21; N, 10.99; Found: C, 34.62, H, 4.14,; N, 11.12%

N^3-DL-3-trans-methoxycarbonyloxirane-2-carbonyl-L-2,3-diaminopropanoic acid trifluoroacetate **8**. This compounds was prepared from **18** as described for **5**. Yield 340 mg, 89% as an amorphous powder, $[\alpha]_{578} = -16.2°$ (c=1, MeOH). Anal. Calc. for $C_8H_{12}N_2O_8CF_3COOH$: C, 31.65; H, 3.45; N, 7.40; Found C, 31.54; H, 3.34; N, 7.38%.

Enzyme purification

Glucosamine-6-phosphate synthase was purified from *Saccharomyces cerevisiae* Y 104 by the method of Milewski *et al.*[8] Protein was determined by the method of Lowry *et al.* with modifications as reported by Layne.[9]

Determination of glucosamine-6-phosphate synthase activity

The standard incubation mixture contained: 15 mM D-fructose-6-phosphate, 10 mM L-glutamine, 1 mM EDTA, 50 mM potassium phosphate buffer (pH 6.5), inhibitor at appropriate concentration, 0.005–0.01 mg ml^{-1} of enzymatic protein in a total volume of 2 ml. In some kinetic experiments, concentrations of glutamine were variable. The mixtures were incubated at 37°C for 30 min. Every 10 min 0.5 ml samples were withdrawn and the reaction was stopped by heating at 100°C for 1 min. The concentration of glucosamine-6-phosphate synthase was determined by the modified Elson-Morgan procedure.[10]

Inactivation of glucosamine-6-phosphate synthase

Incubation mixtures containing 1 mg of protein, 50 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 15 mM D-fructose-6-phosphate and inactivators at various

concentrations in a total volume of 1 ml, were incubated at 25°C. To follow inactivation of the enzyme, 200 μl aliquots were withdrawn from the mixtures and applied at the tops of small, 1 ml columns packed with Sephadex G-25 (equlibrated with the 50 mM potassium phosphate buffer pH 6.5) and centrifuged (500 × g for 1 min at 4°C). Under these conditions the unbound inhibitor was separated from the enzyme and the protein was recovered in clean test tubes. Appropriate effluent aliquots were used for the determination of the residual enzyme activity using the standard assay method.

RESULTS AND DISCUSSION

Synthesis

The preparation of products **5–11** was performed using standard synthetic procedures. Thus, N^2-tert-butoxycarbonyl-L-2,3-diaminopropanoic acid[6] was coupled with monoethyl DL, D and L-*trans*-epoxysuccinate or monomethyl DL-*trans*-epoxysuccinate[7] applying the active esters method (N-hydroxysuccinimide esters) to give **12, 13, 14** and **18**. The ethyl esters **12, 13** and **14** were reacted with ammonia, methylamine and dimethylamine solutions at low temperature (0–5°C) to afford **15, 16, 17, 19** and **20** which were deprotected with trifluoroacetic acid to give **5, 6, 7, 8, 10** and **11** respectively as their hygroscopic salts. It should be noted that **5, 8–11** were prepared as diastereomeric mixtures since racemic *trans*-epoxysuccinic acid was used as a starting material.

Inhibitory activity

All of the obtained compounds were tested as inhibitors of glucosamine-6-phosphate synthase from *Saccharomyces cerevisiae*; their IC_{50} values are summarized in Table 1. The data confirm that the presence of the primary amide in **5** and **6** is important for the inhibitory activity. Secondary and tertiary amides **10** and **11** were completely inactive. On the other hand, the methyl ester derivative **8** was found to inhibit glucosamine-6-phosphate synthase at a higher IC_{50} value than the primary amides **5** and **6** and was more effective than the ethyl ester **9**. However, the esters **8** and **9** were significantly less potent inhibitors than the primary amide derivatives **5** and **6**. Compounds **5–11** were also tested as inactivators of the enzyme. Incubation of *S. cerevisiae* glucosamine-6-phosphate synthase with compounds **5–8**, in the absence of glutamine led to irreversible inactivation of the enzyme. The time course of inactivation caused by compound **6** is shown in Figure 1. When values for the apparent rate constants of inactivation (k_{app}), calculated from the slope of lines in Figure 1, were plotted against inactivator concentration, a hyperbolic curve was obtained (not shown). Such a pattern is consistent with the formation of a reversible complex before inactivation (equation 1):

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E^*I] \overset{k_2}{\rightarrow} EI^* \qquad (1)$$

TABLE 1
Inhibition of glucosamine-6-phosphate synthase from *S. cerevisiae* by compounds **5–11**

Compound	Concentration (μM) causing 50% inhibition of enzyme activity
5	870±80
6	600±40
7	>8000
8	2800±220
9	6100±180
10	>8000
11	>8000

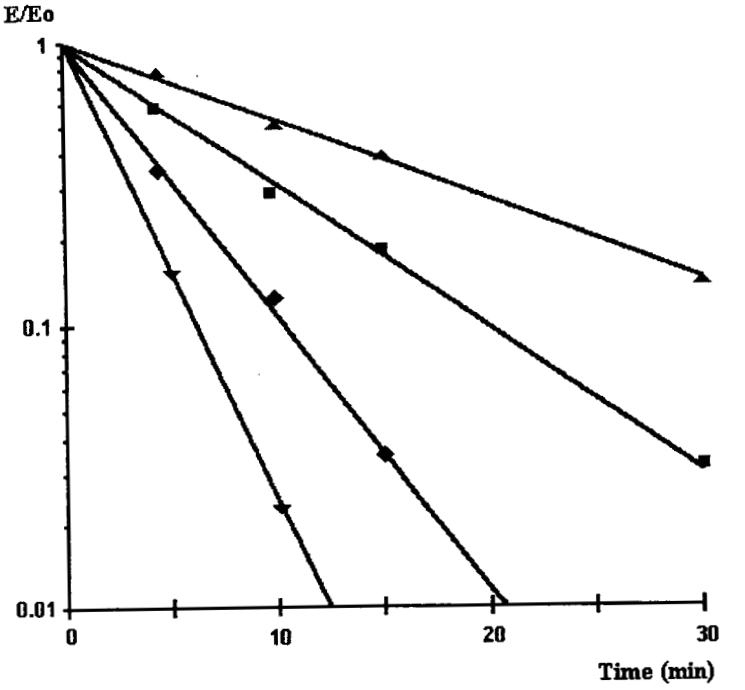


FIGURE 1 Inactivation ofglucosamine-6-phosphate synthase from *S. cerevisiae* Y 104 by compound **6** at concentrations of 62.5 μM (▲), 125 μM (■), 250 μM (♦), 1000 μM (▼). Protein concentration 0.04 mg/ml.

TABLE 2
Kinetic parameters of inactivation of glucosamine-6-phosphate synthase from *S.cerevisiae* by compounds **5–8***

Compound	K_{inact} (μ)M	T (min)	k_2 (min^{-1})	k_2/K_{inact} ($M^{-1}*s^{-1}$)
5	910	1.95	0.355	7
6	385	1.3	0.533	23
7	16000	2.25	0.308	0.3
8	7000	0.85	0.815	2

*compounds **9–11** did not inactivate the enzyme.

[E*I] is the enzyme-inhibitor complex and EI* is the irreversibly modified enzyme. Assuming that [I]≫[E] and that the reversible complex is at all times in equilibrium with enzyme and inhibitor, the equation derived by Meloche[11] can be applied (equation 2):

$$\tau = 1/[1] \times (T \times K_{inact}) + T \quad (2)$$

where K_{inact} is the inactivation constant, τ is a particulate half-time of inactivation and T is the minimal inactivation half-time at infinite inhibitor concentration. Values of K_{inact}, T and k_2 were calculated from a linear plot of inactivation half-times versus reciprocal of inactivator concentration. Kinetic parameters of inactivation for compounds **5–8** are summarized in Table 2. Since the calculated values of apparent second-order rate constants (k_2/K_{inact}) were much lower than k_1 (commonly at least 10^7 M^{-1} s^{-1} for substrates), the quasi-equlibrium assumption for the kinetic two-step model of inactivation is fully justified.[12]

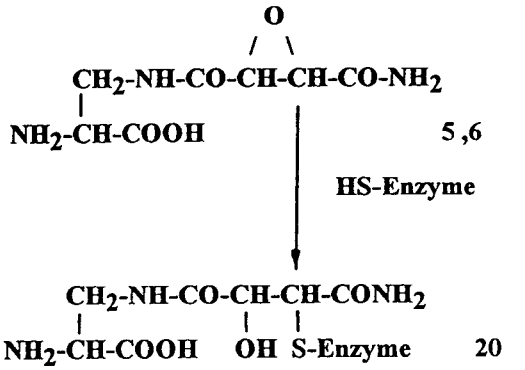
It is apparent from inspection of Table 2, containing the kinetic parameters for the most active inhibitors that the lowest value of K_{inact} was observed for the amide derivative **6** with the 2**R**, 3**R** configuration for the epoxy acid. Since K_{inact} expresses the affinity of an inhibitor for the enzyme active site,[13] compound **6** in contrast to compounds **5, 8** and especially **7** (with 2**S**, 3**S** configuration of epoxy acid) exhibits the highest affinity for the enzyme active site suggesting that there is a steric effect involved in binding to the active site. Although the reactivities of inhibitors as measured by their k_2 values were similar and compound **8** showed an even higher reactivity than compound **6**, their inactivation ability expressed by the ratio of k_2/K_{inact}[11] was seen to decrease in the order **6** > **5** > **8** > **7**. The ratio of K_i (inhibition constant) to K_m for glutamine as a measure of the affinity of the inhibitor to the active site of the enzyme in comparison to glutamine also demonstrated about one order of magnitude higher affinity of compound **6** to the active site of enzyme than glutamine itself (Table 3). Although the molecular mechanism of inactivation of glucosamine-6-phosphate synthase by the synthesized epoxy compounds was not established, it might be suggested that the epoxy ring is opened as a result of S-alkylation of the active site cysteine

TABLE 3
Competitive inhibition of glucosamine-6-phosphate synthase from *S. cerevisiae* by epoxy — compounds **5**, **6** and **8**

Inhibitor	K_i (μM)	K_i/K_m Glu
5	95	$1.73*10^{-1}$
6	40	$7.27*10^{-1}$
8	285	$5.18*10^{-1}$

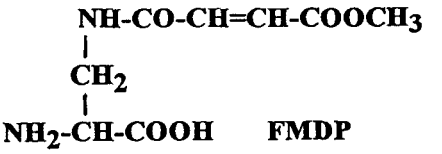
K_m for L-glutamine was $5.5*10^{-4}$M.

present in glucosamine-6-phosphate synthase. The epoxysuccinyl compounds may be attacked by the nucleophilic Cys-1 thiol of the enzyme at either the C-3 or the C-2-carbon atom of the epoxy acid. The naturally occuring epoxysuccinate E-64[14] and its derivatives[15–18] are known as rapid and irreversible inhibitors of many thiol proteases. Spectroscopic[19] and crystallographic[20–21] studies have demonstrated that the Cys anion of papain attacks the epoxide C-3 carbon atom to produce a covalent adduct. If the same C-3 carbon atom in



epoxy compounds **5–11** was attacked by the Cys-1 thiol group, covalent adducts like **20** should be formed. However, our data showed that not all the tested compounds may form such adducts, thus a steric factor should be taken into account. It is highly likely that in the case of the substituted amide (**10**, **11**) and ester derivatives (**8**, **9**) the Cys-l thiol group may not react with the oxirane ring at the C-3 carbon atom of the *trans*-epoxysuccinate, presumably due to the sterically congested environment around the carboxyl group. Therefore, inactivation of glucosamine-6-phosphate was not observed for those compounds. It should be noted that epoxysuccinoyl compounds are entirely stable toward attack by thiols in a nonenzymatic reaction. In our experiment, when compounds **5** and **9** were incubated with equimolar amounts of reduced glutathione in phosphate buffer pH 7.25 at room temperature for 2 hours, no reactions were observed. These results indicate a general low chemical reactivity of the

examined epoxy compounds in regard to nucleophilic attack by the SH group under physiological pH values. This property is advantageous and it allows the prediction, that in the physiological environment of the body fluids, epoxy inhibitors would not react nonspecifically with the various thiol components present in abundance in these fluids. On the other hand the inhibitory activity of compounds **5** and **6** indicates that the Cys-1 thiol group — oxirane ring interaction is possible upon binding to the enzyme active site due to the resulting proximity and orientation effects. Under the same conditions N^3-4-methoxyfumaroyl-L-2,3-diaminopropanoic acid (FMDP), a known inhibitor of glucosamine-6-phosphate synthase,[8] rapidly formed



FMDP

a covalent bond with glutathione. The comparison of the nonspecific chemical reactivity of epoxy compounds and Michael type acceptors as enzyme inhibitors points to the former compounds as more valuable inhibitors of glucosamine-6-phosphate synthase.

In conclusion, compound **6** with **(R,R)** configuration of the *trans*-epoxysuccinic acid was found to be the most effective inhibitor tested in this study and it may act as an affinity labeling agent of the enzyme Cys-l thiol group by nucleophilic oxirane ring opening at the C-3 carbon atom of the *trans*-epoxysuccinic acid.

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