New lysine chromogenic substrates for trypsin

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Eight new direct chromogenic lysine substrates, namely N^{α}*-benzyloxycarbonyl-L-lysine 3,5-dinitroanilide hydrochloride (L-ZLDA·HCl),* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitroanilide hydrochloride (L-ZLNA·HCl),* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5-bromoanilide hydrochloride (L-ZLNBA·HCl),* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5-chloroanilide hydrochloride (L-ZLNCA·HCl),* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5-fluoroanilide hydrochloride (L-ZLNFA·HCl),* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5-iodoanilide hydrochloride (L-ZLNIA·HCl),* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5-(methylsulphonyl) anilide hydrochloride (L-ZLNMA·HCl) and* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5-(trifluoromethyl) anilide hydrochloride (L-ZLNTA·HCl) were synthesized by the direct condensation of* N^{α}*-benzyloxycarbonyl,* N^{ϵ}*-t-butyloxycarbonyl-L-lysine and 3-nitro-5X-substituted aniline, where (*$X = F$, Cl, Br, I, H, NO_2, CF_3 *or* SO_2CH_3*), using dicyclohexylcarbodiimide (DCC) as a coupling reagent. The resulting product,* N^{α}*-benzyloxycarbonyl-*N^{ϵ}*-t-butyloxycarbonyl-L-lysine-3-nitro-5X-substituted anilide was treated with 2 M HCl in dioxane at room temperature to give* N^{α}*-benzyloxycarbonyl-L-lysine 3-nitro-5X-substituted were found to be stable to relatively wide variations of temperature and pH. Trypsin-catalysed hydrolysis of these substrates at 37°C proceeded at significantly different rates in the following order: L-ZLNIA·HCl> L-ZLNBA·HCl> L-ZLNCA·HCl> L-ZLNA·HCl> L-ZLNTA·HCl> L-ZLNFA·HCl> L-ZLNMA·HCl> L-ZLDA·HCl. Kinetic and thermodynamic parameters such as* K_m, V_{max}, k_{cat}, E_a, ΔH *and* ΔS *were determined for the trypsin-catalysed hydrolysis of each substrate. A Hammett plot of the catalytic rate constants gave a straight line with a* ρ *value of* -1.79 *at 37°C thus indicating that electron withdrawing substituents inhibit the trypsin-catalysed hydrolysis of the new lysine substrates.*

Keywords: Trypsin; chromogenic lysine substrates; hydrolysis; catalytic rate constants

Introduction

The introduction of chromogenic compounds as substrates for enzymes has considerably simplified the procedure for the determination of enzymatic activities. The first set of chromogenic substrates include α-naphthylesters and α-naphthylamides of the α-amino acylated amino acids in which the α-naphthol or the α-naphthylamine released is coupled to a diazonium salt to produce an azo dye[1-3]. These substrates are indirect chromogenic substrates since post-incubation processing of the end product is required. Direct chromogenic ester and amide substrates such as *p*-nitrophenyl esters and *p*-nitroanilides of α-amino acylated amino acids offer the advantage that post-incubation processing of the end product is not required since the progress of the enzymatic reaction can be continuously monitored by measuring the coloured product (*p*-nitrophenol or *p*-nitroaniline) spectrophotometrically. The chromogenic ester substrates such as *p*-nitrophenyl esters are not as suitable as the *p*-nitroanilide substrates because of the appreciable degree of autolysis in neutral or alkaline medium[4].

The rate of hydrolysis of amino acid substrates can be significantly affected by structurally modifying the side chain, α-amino group or the carboxyl group[5-7]. Structural modification brought about by introducing substituents on the carboxyl group is particularly useful in studying the electronic effects on the enzyme activity. In the trypsin-catalysed hydrolysis of an anilide substrate, the acylation step has been reported to be rate limiting[8,9] hence structural modification in the leaving group of the anilide would produce an observable change in the steady state rate of the hydrolysis of the substrate. This advantage is non-existent for ester substrates since it has been shown by Caplow and Jencks[9] that the deacylation step is rate limiting.

In this study, the effect of structural modification of the substrate on the tryptic activity was investigated with eight new chromogenic lysine substrates, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5X-anilide hydrochloride (X = H, CF_3, NO_2, SO_2CH_3, F, Cl, Br or I). This series of substrates is very suitable for studying electronic effects on trypsin activity because substituents in the *meta* position are remote from the reaction centre thereby reducing steric effect to a minimum. Also the coloured amine liberated on enzymic hydrolysis can easily be monitored by spectrophotometric method. The same series of lysine substrates can be suitable as substrates for plasmin since it is known that plasmin prefers substrates

Abbreviations: L-ZLNA·HCl, N^{α}-benzyloxycarbonyl-L-lysine-3-nitroanilide hydrochloride; L-ZLNBA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5-bromoanilide hydrochloride; L-ZLNCA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5-chloroanilide hydrochloride; L-ZLNFA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5-fluoroanilide hydrochloride; L-ZLNIA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5-iodoanilide hydrochloride; L-ZLNMA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5-(methylsulphonyl) anilide hydrochloride: L-ZLNTA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3-nitro-5-(trifluoromethyl) anilide hydrochloride; L-ZLDA·HCl, N^{α}-benzyloxycarbonyl-L-lysine 3,5-dinitroanilide hydrochloride.

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containing a lysine side chain unlike thrombin and kallikrein which preferentially hydrolyse substrates with an arginine side chain[10].

Experimental

All the reagents used throughout this investigation were of analytical grade.

The ultraviolet and visible absorption spectra of the chromogenic substrates and the 5-substituted-3-nitroaniline were measured with a Zeis PMQ 3 spectrophotometer attached to a Haake F3 thermostatically regulated waterbath. The optical rotations of all the substrates were measured with a Perkin-Elmer recording polarimeter, model 241. Elemental analyses were carried out on a Yanagimoto CHN corder, model MT-2, at the Institute of Pharmaceutical Sciences of Hokkaido University, Sapporo, Japan. Melting points for all samples were determined on Reichert melting point apparatus.

Buffers. 0.05 M Tris (hydroxymethyl) amino methane–HCl and 0.05 M glycine–NaOH buffer solutions each containing 0.02 M calcium chloride at pH values ranging from 7.0 to 10.0 were used.

Enzyme. Twice crystallized bovine pancreatic trypsin with a specific activity of 180 units/mg protein was purchased from P-L Biochemicals Milwaukee, USA. The trypsin specific activity was determined using L-tosyl arginine methyl ester hydrochloride (L-TAME · HCl) as a substrate according to the Worthington procedure[11]. The concentrations of enzyme used ranged from 17 to 60 μg/ml.

N$^{\alpha}$-Benzyloxycarbonyl-L-lysine. The lysine derivative was prepared according to the procedure of Costopanagiots *et al.*[12].

N$^{\alpha}$-Benzyloxycarbonyl-N$^{\varepsilon}$-t-butyloxycarbonyl-L-lysine. This lysine derivative was prepared according to the procedure of Schwyzer and Rottel[13].

3-Nitro-5X-anilines. 3,5-Dinitroaniline, 3-nitro-5-chloroaniline, 3-nitro-5-bromoaniline, 3-nitro-5-iodoaniline, 3-nitro-5-fluoroaniline, 3-nitro-5-(methylsulphonyl) aniline and 3-nitro-5-(trifluoromethyl) aniline were prepared according to the procedure reported by Emokpae *et al.*[14].

Lysine chromogenic substrates, N$^{\alpha}$-benzyloxycarbonyl-L-lysine-5-substituted 3-nitroanilide hydrochloride. The 3-nitro-5X-aniline (X = H, CF_3, NO_2, SO_2CH_3, F, Cl, Br and I) was directly coupled to N^{α}-benzyloxycarbonyl, N^{ε}-t-butyloxycarbonyl-L-lysine by a modification of the procedure reported by Erlanger *et al.*[4] for the synthesis of N^{α},N^{ε}-dibenzyloxycarbonyl-L-lysine-*p*-nitroanilide. Dicyclohexylcarbodiimide (DCC), 0.4 g (1.98 mmol), dissolved in 10 ml of dry dioxane was mixed with 20 ml of dry dioxane solution containing 0.5 g (1.3 mmol) N^{α}-benzyloxycarbonyl, N^{ε}-t-butyloxycarbonyl-L-lysine and 1.3 mmol 5-substituted-3-nitroaniline. The mixture was stirred for 24 h at room temperature. Glacial acetic acid, 0.04 ml (0.68 mmol), was then added to destroy the excess DCC before filtering off the dicyclohexylurea. The filtrate was concentrated under reduced pressure at 35°C and the residue taken up in ethyl acetate, washed successively with 10% citric acid, water, 4% sodium bicarbonate, water and dried over anhydrous sodium sulphate. The ethyl acetate was removed under reduced pressure at 28°C and the resulting syrup dissolved in 10 ml of 2 M HCl in dry dioxane and stirred at room temperature for 1 h. N^{α}-benzyloxycarbonyl-L-lysine 5-substituted-3-nitroanilide hydrochloride was precipitated by the addition of 200 ml dry ether. The precipitate separated by centrifugation was dried and recrystallized from dry dioxane–ether (1:3) mixture. The m.p. and optical rotation of each chromogenic lysine substrate are listed in *Table 1*.

Enzymatic activity determination

The initial rates of trypsin-catalysed hydrolyses of the 5-substituted-3-nitroanilide substrates were determined by continuous measurement of free 5-substituted-3-nitroaniline while the reaction proceeded in a cuvette inserted in a PMQ 3 automatic spectrophotometer, as in Method I of Erlanger *et al.*[4]. The concentration of the substituted aniline was determined at 410 nm, at which wavelength all the chromogenic substrates have no contribution to the absorbance. The reaction conditions are given in the tables and legends to figures.

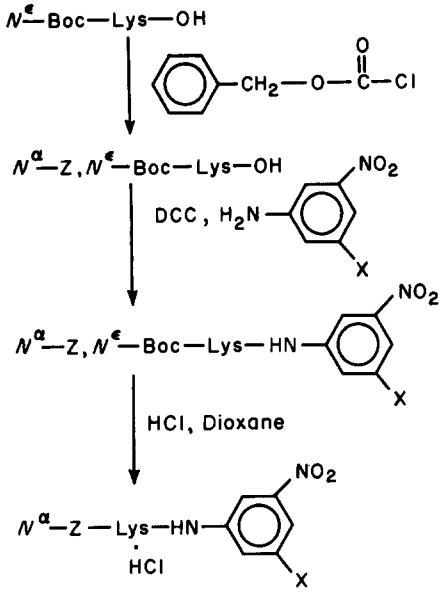
Results and discussion

Synthesis of N^{α}-amino acylated lysine chromogenic substrates has always been difficult because of the reactivity of the ε-amino group of lysine which makes the selective protection of the α-amino group difficult. In this study, the N^{α}- and N^{ε}-amino groups of lysine were selectively protected by employing the t-butyloxycarbonyl group to block the N^{ε}-amino group and benzyloxycarbonyl group to protect the α-amino group. The synthetic route to the chromogenic substrates is summarized in Scheme 1. In order to minimize procedural difficulties, avoid extensive purification and obtain maximum yield, a method which involves the direct condensation of N^{α}-benzyloxycarbonyl-N^{ε}-t-butyloxycarbonyl-L-lysine and 5-substituted-3-nitroaniline using dicyclohexylcarbodiimide (DCC) as a coupling agent was chosen after a survey of possible synthetic routes. A slight excess of DCC was found necessary to bring the coupling reaction close to 100% at room temperature. The two amino protecting groups, benzyloxycarbonyl and t-butyloxycarbonyl were found to be effectively stable under the conditions used for the synthesis. The deblocking of the N^{ε}-amino function was smoothly achieved without any significant effect on the benzyloxycarbonyl group at the N^{α}-position

Table 1

Substrate	$[\alpha]_D^{25}$ C = 1 in EtOH	M.p. (°C)
L-ZLNIA · HCl	−19.6°	169–171
L-ZLNBA · HCl	−21.2°	210–212
L-ZLNCA · HCl	−27.4°	183–185
L-ZLNA · HCl	−33.6°	207–209
L-ZLNFA · HCl	−32.6°	184–186
L-ZLNTA · HCl	−27°	177–179
L-ZLNMA · HCl	−35.2°	180–182
L-ZLDA · HCl	−34.59°	192–194

by treating the N^{α}-benzyloxycarbonyl-N^{ε}-t-butyloxycarbonyl-L-lysine 5X-3-nitroanilide with 2 M HCl in dry dioxane at room temperature for 1 h. The desired chromogenic substrate was readily precipitated with anhydrous ether and recrystallized with dioxane–ether mixture. The results show that the elemental analyses of the new lysine substrates are in good agreement with theoretical values. The synthetic substrates were sufficiently soluble in water to permit kinetic studies without the addition of organic solvents such as dimethylformamide. These lysine substrates showed no absorbance at 410 nm, a wavelength at which the concentration of the substituted aniline released was estimated spectrophotometrically. The molar absorption coefficients of the amines at 410 nm are listed in *Table 2*.



Scheme 1 Synthetic route for N^{α}-benzyloxycarbonyl-L-lysine 5-substituted-3-nitroanilide hydrochloride

Table 2 Molar absorption coefficients of the 5-substituted-3-nitroanilines at 410 nm

Substituted aniline	A_{410} ($cm^2\ mol^{-1}$)
5-Fluoro-3-nitroaniline	630
5-Trifluoromethyl-3-nitroaniline	720
5-Chloro-3-nitroaniline	735
3-Nitroaniline	780
5-Bromo-3-nitroaniline	810
5-Iodo-3-nitroaniline	840
5-Methylsulphonyl-3-nitroaniline	1080
3,5-Dinitroaniline	1560

The pH profiles of the trypsin-catalysed hydrolysis of the lysine chromogenic substrates show broad curves with optimum at pH 8.2 to 8.5. This pH range remains unchanged on varying the ionic strength of the reaction medium by the addition of increasing amounts of sodium chloride up to 200 mM. A range of 8 to 8.2 has been reported for the optimum pH of trypsin-catalysed hydrolysis of other anilides[15].

The initial rates of the trypsin catalysed hydrolysis of all the lysine substrates were found to be linear with trypsin over a tenfold range up to 60 μg/ml.

The relative rates of hydrolysis are presented in *Table 3*. The observed order is L-ZLNIA·HCl> L-ZLNBA·HCl> L-ZLNCA·HCl> L-ZLNA·HCl> L-ZLNTA·HCl> L-ZLNFA·HCl> L-ZLNMA·HCl> L-ZLDA·HCl. Using the linear regression analysis on a Hanes plot the classical constants k_{cat}, and K_m were determined and listed in *Table 4*. As expected, the order of

Table 3 Relative rates of trypsin-catalysed hydrolysis of the various lysine substrates[a]

Substrate	Relative rate
L-ZLDA·HCl	1.0
L-ZLNMA·HCl	1.13
L-ZLNFA·HCl	2.09
L-ZLNTA·HCl	2.17
L-ZLNA·HCl	2.52
L-ZLNCA·HCl	2.65
L-ZLNBA·HCl	3.39
L-ZLNIA·HCl	12.0

[a] Initial substrate concentration: 3×10^{-3} M in 50 mM Tris–HCl buffer, pH 8.5, containing 20 mM $CaCl_2$. The enzyme reaction was carried out at 37°C with an enzyme concentration of 1.35 μM

Table 4 Kinetic parameters for trypsin-catalysed hydrolysis of L-ZLDA·HCl, L-ZLNA·HCl, L-ZLNBA·HCl, L-ZLNCA·HCl, L-ZLNFA·HCl, L-ZLNIA·HCl, L-ZLNMA·HCl and L-ZLNTA·HCl[a]

	Parameters					
	k_{cat}, s^{-1}			K_m (mM)		
Substrate	25°C	30°C	37°C	25°C	30°C	37°C
L-ZLDA·HCl	0.11	0.13	0.23	0.65	0.66	1.0
L-ZLNMA·HCl	0.15	0.17	0.26	0.75	0.85	0.98
L-ZLNFA·HCl	0.17	0.20	0.48	0.79	0.82	1.25
L-ZLNTA·HCl	0.21	0.29	0.50	1.05	1.47	1.90
L-ZLNA·HCl	0.22	0.36	0.58	1.30	1.25	1.60
L-ZLNCA·HCl	0.23	0.41	0.61	0.85	1.27	1.10
L-ZLNBA·HCl	0.33	0.44	0.78	0.40	0.55	0.68
L-ZLNIA·HCl	0.46	0.78	2.76	0.45	0.74	1.75

[a] Initial substrate concentration: 330–1000 μM in 50 mM Tris–HCl buffer, pH 8.5, containing 20 mM $CaCl_2$. The reaction was carried out with an enzyme concentration of 1.35 μM

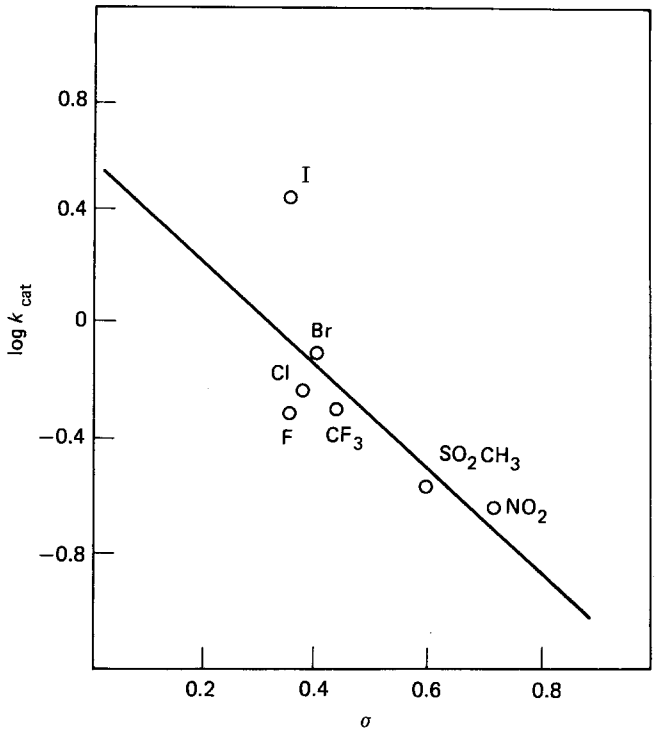


Figure 1 The plot of log k_{cat} *versus* σ at 37°C

k_{cat} values for the various substrates is the same as the observed values of the relative rates of hydrolysis listed in *Table 3*. The sequence can be explained by considering the mechanism of the trypsin-catalysed hydrolysis of anilides. It is now well known that trypsin hydrolysis proceeds via the acyl-enzyme intermediate with an overall mechanism which can be represented by the equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

where k_2 and k_3 are the first order rate constants for the acylation and deacylation steps respectively; P_1 and P_2 are products[16,17]. In the case of trypsin-catalysed hydrolysis of anilide substrates, the acylation step has been reported by Bender *et al.*[16] to be the rate limiting step. The influence of the electronic effects of substituents in the substrates is shown by the Hammett ρ-σ plot for the first order rate constants, k_{cat} (k_2). The plot shown in *Figure 1* was linear with a ρ value of −1.79. Inagami *et al.*[18] have shown that in the chymotrypsin-catalysed hydrolysis of substituted anilides of *N*-acetyl-L-tyrosine, the ρ-σ plot for k_{cat} gave a ρ value of −2.0. Also Sager and Parks[19] have reported a ρ value of −1.63 for chymotrypsin-catalysed hydrolysis of some anilide substrates. Conventionally it is known that the negativity of ρ suggests that the acylation step is inhibited by electron withdrawing substituents in the anilide substrates. Accordingly, it is expected that the rate of hydrolysis of L-ZLDA·HCl< L-ZLNMA·HCl< L-ZLNTA·HCl< L-ZLNA·HCl since the negative inductive effect sequence is $NO_2 > SO_2Me > CF_3 > H$. This expected order of reactivity was obtained as evident from the results in *Tables 3* and *4*. Contrary to expectation, the halogen substituents enhanced the rate of hydrolysis. Ordinarily, the halogens are known to be more electron withdrawing than hydrogen and should therefore be more deactivating than hydrogen in this series of substrates. The order of reactivity L-ZLNFA·HCl<L-ZLNCA·HCl< L-ZLNBA·HCl<L-ZLNIA·HCl follows the expected sequence of negative inductive effect (−I effect) of the groups, F>Cl>Br>I. Besides the −I effect, the halogen substituent had lone pairs of electrons which can be relayed into positions *ortho* and *para* to it and this effect can be transmitted by second-order relay to the nitrogen of the amino group. This resonance effect (+R effect) is too weak to counteract the deactivation caused by the −I effect of the halogen substituent. However, at the point of attack, the electromeric effect (+E effect) is brought into play on the demand of the attacking reagent and the electron densities at the *ortho* and *para* positions are raised with a concomitant increase in the electron density on the amino nitrogen atom by second order relay. This argument was invoked to explain the *ortho–para* directing influence of the halogens in aromatic electrophilic substitution.

The Arrhenius plots for the activation energies were constructed according to the method of Gorgani and Meisami[20]. From the slope of the line, the activation energies, E_a, for the enzyme reactions were determined and the values obtained for the different lysine substrates are listed in *Table 5*. From the activation energies, all other thermodynamic constants were determined. A plot of ΔH against ΔS gave a straight line shown in *Figure 2*. On determining ρ values at three different temperatures, these values were found to vary inversely with the temperature as shown in *Figure 3*. The variation of ρ with temperature has also been observed by Bolton and Hall[21] in the spectrophotometric determination of the thermodynamic acidity constants of *meta*-substituted anilinium ions. For this non-enzymic reaction series, a substituent change causes ΔH and ΔS to vary in opposite directions and a plot of ΔH against ΔS showed a high degree of linear proportionality with the slope corresponding to the isokinetic temperature, β, which was found to be −263.5 K. Bolton and Hall[21] rationalized their results by assuming that for any reaction series, the substituent induced changes on the thermodynamic functions measured at temperature T are related to the Hammett parameters by the equation:

$$\rho = \frac{\delta \Delta S}{2.303 R \sigma}\left(1 - \frac{\beta}{T}\right)$$

This in effect means that there is an inverse relationship between ρ values and temperature for a reaction which has a negative isokinetic temperature. From the plot of ρ against $1/T$ shown in *Figure 3*, the variation of ρ with temperature follows the proposition of Bolton and

Table 5 Values of the activation energy (E_a) obtained for the trypsin-catalysed hydrolyses of the lysine substrates

Substrate	E_a (kcal/mol)
L-ZLDA·HCl	8.03
L-ZLNMA·HCl	8.19
L-ZLNCA·HCl	9.02
L-ZLNBA·HCl	9.56
L-ZLNTA·HCl	9.64
L-ZLNA·HCl	10.72
L-ZLNFA·HCl	11.31
L-ZLNIA·HCl	12.49

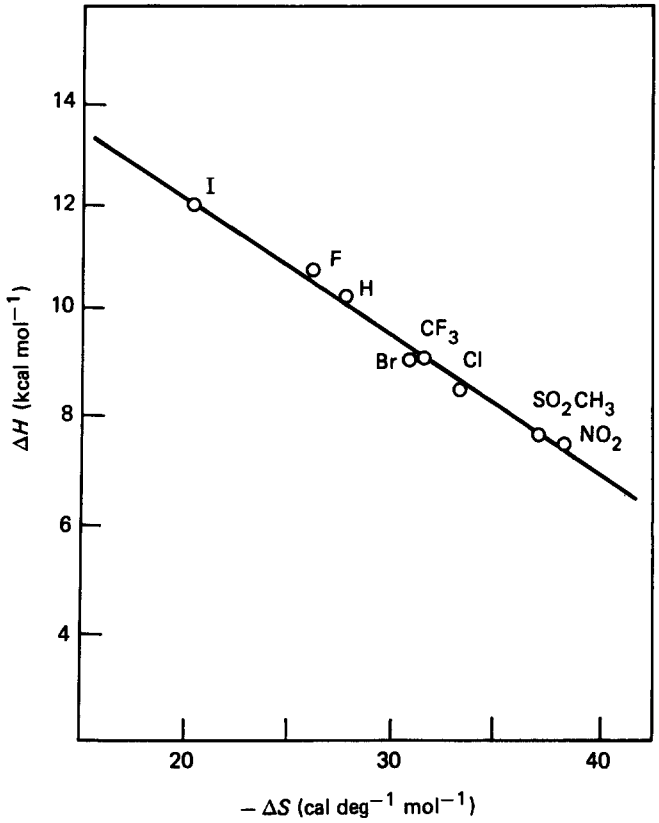


Figure 2 The plot of ΔH *versus* $-\Delta S$ at 25°C to determine the isokinetic temperature, β

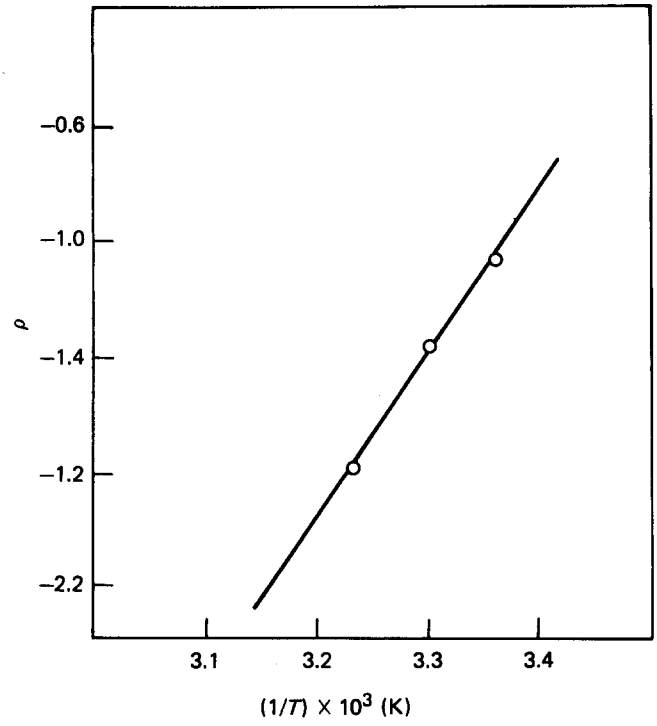


Figure 3 Variation of ρ with $1/T$

Hall[21]. However, this relationship contrasts with the findings of Inagami *et al.*[18] who reported that ρ varies directly with temperature.

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