Tetrahedron Vol 49, No. 44, pp. 10099-10102, 1993
Printed in Great Britain

0040-4020/93 \$6.00+.00
© 1993 Pergamon Press Ltd

Electronic Substituent Effects in the Nitrilase-Catalyzed Hydrolysis of Para-Substituted Benzyl Cyanides

Shimona Geresh,[1]* Yakir Giron,[2] Ygal Gilboa,[1] and Robert Glaser[2]

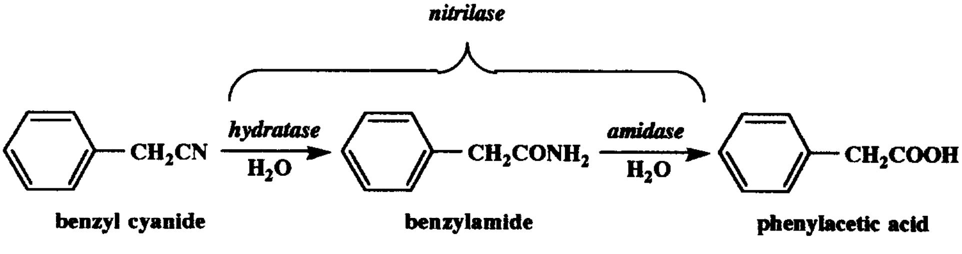
[1]The Institutes for Applied Research, and [2]Department of Chemistry
Ben-Gurion University of the Negev, Beer-Sheva 84110, ISRAEL

(*Received in UK* 9 *July* 1993; *accepted* 27 *August* 1993)

Abstract: The initial rates of the nitrilase (Novo)-catalyzed hydrolysis of a series of para-substituted benzyl cyanides (R = NO_2, Cl, OCH_3, OH, NH_2) were found to be susceptible to the nature of the para-substituent of the substrate and a Hammett-type linear free energy correlation was observed with $\rho = 0.96$ In a separate study, effective solubilization of substituted benzyl cyanide substrates having electron-donating groups (OH, NH_2, OCH_3) was achieved upon mixing with β-cyclodextrin to form 1 1 mol ratio inclusion complexes, but para-substituted benzyl cyanides with electron-withdrawing groups (C*l*, NO_2) were not fully solubilized under the same conditions In addition, it was shown that the presence of β-cyclodextrin not only had no inhibitory effect on the enzyme activity, but it actually increased the initial rate of hydrolysis of the unsubstituted benzyl cyanide β-cyclodextrin inclusion complex However, the initial rates of hydrolysis were observed to be smaller when β-cyclodextrin was added to the para-substituted benzyl cyanides

INTRODUCTION

Nitrilases from various sources have been used to hydrolyze aliphatic and aromatic nitriles under mild conditions [1-4] An immobilized nitrilase preparation (SP) from Novo Industri A/S (Denmark) has been reported for the enzymatic hydrolysis of acyclic, cyclic and heterocyclic nitriles and dinitriles or nitriles that contain base- or acid-sensitive groups [2,3,5]



Scheme 1

The Novo-nitrilase is, in fact, a preparation of two enzymes, a hydratase (which catalyzes the conversion of the nitrile to the amide) and an amidase (which catalyzes the hydrolysis of the amide to the corresponding acid, see Scheme 1) The specific activities of the two enzymes differ (the hydratase having a much higher activity) While the optimal reaction conditions (pH and temperature) also differ for the two enzymes, it is difficult to stop the enzymatic reaction at the amide stage if so desired Thus, it is not practical to apply Scheme 1 as a synthetic route to amides using the Novo nitrilase.

Benzyl cyanide (phenylacetonitrile) and ring-substituted benzyl cyanides are versatile building blocks in organic chemistry due to the functionality of the CN group. Benzyl cyanide itself is known to be a suitable substrate for nitrilase.[1,2] In this paper we report the effect of substituents on the hydrolysis of a series of para-substituted benzyl cyanides catalyzed by immobilized nitrilase [Novo Industri A/S]

RESULTS AND DISCUSSION

Inspection of Figure 1 shows that production of phenylacetic acid is extremely slow at the beginning of the nitrilase catalyzed hydrolysis of benzyl cyanide. This was also found to be true for the para-substituted benzyl cyanide analogues investigated in this study

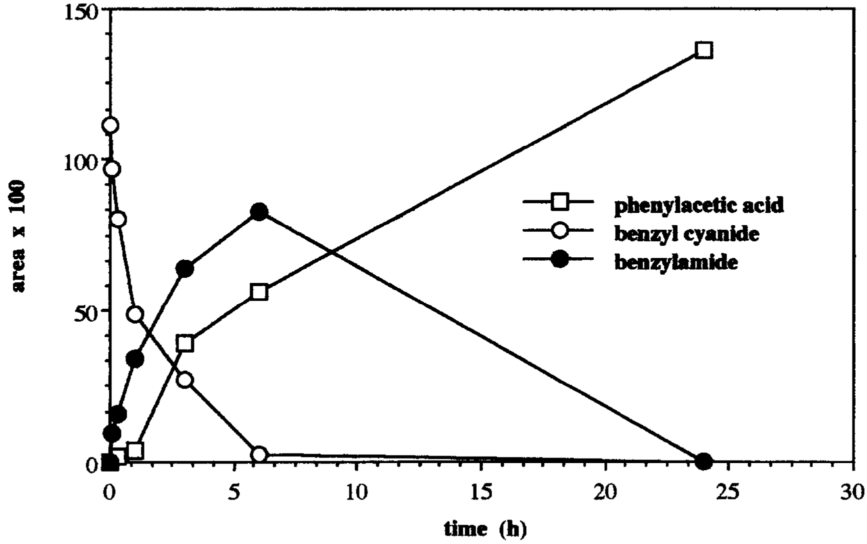


Figure 1. Nitrilase catalyzed hydrolysis of benzyl cyanide and formation of benzylamide and phenylacetic acid products

The initial rates of the hydratase-catalyzed hydrolysis of a series of para-substituted benzyl cyanides were found to be dependent upon the nature of the para-substituent (R = NO_2, Cl, H, OCH_3, OH, NH_2, see Table 1) Linear free-energy relationships in enzyme-catalyzed reactions have been used to facilitate understanding of reactivity, and to compare transition-state structures with those of non-enzymatic reactions[7,8] In addition, Hammett-type analyses have been used to compare enzyme-catalyzed reactions in organic solvents with those in aqueous media[7] The data obtained suggested similar microenvironments for the transition state in the various media.

The results depicted in Fig 2 illustrate a $(7 + \log k_{initial}) = 0\,95\sigma + 1\,21$ correlation between nitrilase catalyzed hydrolysis initial rates and the para-substituent σ-constant [correlation coefficient = 0 98], and thus the electron density at the reaction center is clearly effected by the nature of the para-substituent Since the $\rho = 0\,95$ reaction constant is positive, the reaction is accelerated by electron-withdrawing substituents, and is slowed down by electron-donating substituents It is known that reaction constants decrease when the reaction site is separated from the substituted aryl ring by an increasing number of carbon atoms For example, the reaction constants obtained for the dissociation reaction in aqueous solutions of a homologous series of aromatic carboxylic acids [$ArCOOH$, $ArCH_2COOH$ and $ArCH_2CH_2COOH$] were reported as 1.00, 0.56 and 0.24, respectively.[9] In our case, although only a single methylene group separates the reactive cyanide from the para-substituted aryl group, the nitrilase initial reaction constant value of 0.95 reflects an even stronger susceptibility of the reaction to electronic substituent effects than that of the dissociation of $ArCH_2COOH$. Removal of the methylene spacer between the reactive cyanide and substituted aryl group afforded characteristically higher reaction constants for the base catalyzed hydrolysis of a series of substituted benzonitriles [$\rho = 2.31$ (free $ArC\equiv N$)[10] and 1.93 (coordinated $(NH_3)_5CoArC\equiv N$)[11]]

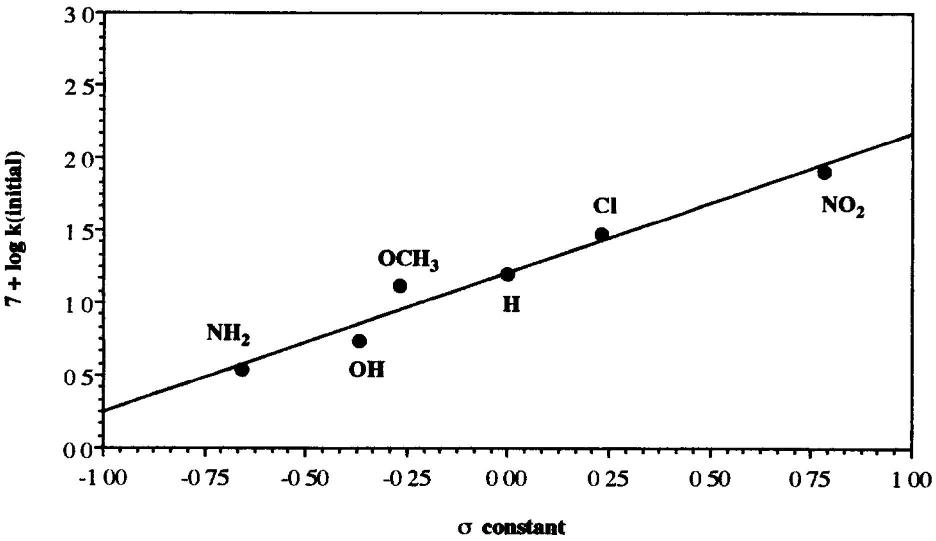


Figure 2 Hammett correlation for the nitrilase-catalyzed hydrolysis of para-substituted benzyl cyanides

Table 1 Initial Rates of Hydratase-Catalyzed Hydrolysis of Various Para-Substituted Benzyl Cyanides[a]

Substituent	σ value	Initial rate/mg enzyme x 10^6
$-NH_2$	-0.66	0.35
-OH	-0.37	0.54
$-OCH_3$	-0.27	1.33
-H	0.00	1.58
-Cl	0.23	2.96
$-NO_2$	0.78	8.13

[a] Reaction conditions as described in the experimental section. The initial rates and the progress of the nitrilase-catalyzed hydrolysis were determined by HPLC analysis of samples withdrawn from the reaction mixture

Organic solvents miscible in water (*e.g.* dimethyl sulfoxide, ethanol, ethylene glycol, acetone) were then used to increase substrate solubility. It was noted that the amidase-catalyzed hydrolysis rate of the respective benzyl cyanides decreased significantly as the proportion of organic solvent in water increased. Recently, Otero *et al.*[6] reported the use of cyclodextrins to solubilize hydrophobic materials as substrates in enzyme-catalyzed reactions Independently we found that effective solubilization was achieved when benzyl cyanide or its para-substituted analogues having electron-donating groups (OH, NH_2, OCH_3) were mixed with β-cyclodextrin (in a molar ratio of 1·1), but that para-substituted benzyl cyanides with electron-withdrawing groups (Cl, NO_2) were not fully solubilized under the same conditions. In addition, it was shown that the presence of β-cyclodextrin not only had no inhibitory effect on the enzyme activity, but it actually increased the initial rate of hydrolysis of the unsubstituted benzyl cyanide·β-cyclodextrin inclusion complex. However, the rates of hydrolysis at the beginning of the reaction (when acid formation is almost nonexistent) were observed to be smaller when β-cyclodextrin was added to the para-substituted benzyl cyanides

EXPERIMENTAL

An aqueous solution of para-substituted benzyl cyanide (about 0.25 mmol) was added to a suspension of immobilized activated nitrilase enzyme [Novo Industri A/S] (about 30 mg) in 0 01 M phosphate buffer, pH 8, in a total volume of 80 ml The reaction mixture was stirred at room temperature with a magnetic stirrer. Samples were withdrawn at intervals, filtered through a 0.45 mμ nylon filter, and analyzed on a Supelco HPLC reverse-phase column by elution with an acetonitrile:water mixture Rates were calculated as the change in substrate concentration with time divided by the amount of enzyme used, and express the apparent specific activity of the enzyme for the particular substrate used

REFERENCES

1 Ingvorsen, K.; Yde, B.; Godtfredsen, S. E.; Tsuchiya, R. T. in *Cyanide Compounds in Biology* CIBA Foundation Symposium, vol.140, Wiley: Chichester, 1988, pp. 16-31
2. Cohen, M. A ; Sawden, J. N.; Turner, J *Tetrahedron Lett.* **1990**, *31*, 7223-7226.
3. Klempier, N.; de Raadt, A.; Faber, K.; Griengl, H. *Tetrahedron Lett.* **1991**, *32*, 341-344
4. Nagasawa, T., Yamada, H. *Pure Appl. Chem.* **1990**, *62*, 1441-1444
5. Hönicke-Schmidt, P.; Schneider, M. P *J. Chem. Soc., Chem. Commun.*, **1990**, 648-650
6 Otero, C ; Cruzado, C.; Ballesteros, A *Appl. Biochem. Biotechnol.*, **1991**, *27*, 185-194
7. Kanerva, L. T.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 6864-6865
8. Kanerva, L. T.; Vilhanto, J ; Pajunen, E.; Euranto, E. K *Acta Chem. Scand.* **1990**, *44*, 489-491
9. Johnson, C. D. *The Hammett Equation*, Cambridge Univ. Press: Cambridge, 1973
10. Zanella, A W.; Berkamp, M A., Second Chemical Conference of the North American Continent, Las Vegas, NV, Aug. 1980, American Chemical Society. Washington, abstr INOR 24
11 Lopez de la Vega, R.; Ellis, W R , Jr.; Purcell, W. L. *Inorg. Chim. Acta* **1983**, *68*, 97-101

ACKNOWLEDGEMENT

We thank Novo Industri A/S, Denmark, especially Ms B Yde and Dr T Hansen, for the generous gift of immobilized nitrilase.