A kinetic-isotope-effect study of catalysis by *Vibrio cholerae* neuraminidase

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Michaelis–Menten parameters for hydrolysis of seven aryl *N*-acetyl α -D-neuraminides by *Vibrio cholerae* neuraminidase at pH 5.0 correlate well with the leaving-group pK_a ($\Delta pK 3.0$; $\beta_{ig}(V/K) = -0.73$, $r = -0.93$; $\beta_{ig}(V) = -0.25$; $r = -0.95$). The β -deuterium kinetic-isotope effect, $\beta_{D_2}(V)$, for the *p*-nitrophenyl glycoside is the same at the optimum pH of 5.0 (1.059 ± 0.010) as at pH 8.0 (1.053 ± 0.010), suggesting that isotope effects are fully expressed with this substrate at the optimum pH. For this substrate at pH 5.0, leaving group ^{18}O effects are $^{18}(V) = 1.040 \pm 0.016$ and $^{18}(V/K) = 1.046 \pm 0.015$, and individual secondary deuterium effects are $\beta_{proRD}(V) = 1.037 \pm 0.014$, $\beta_{proSD}(V) = 1.018 \pm 0.015$, $\beta_{proRD}(V/K) = 1.030 \pm 0.017$,

$\beta_{proSD}(V/K) = 1.030 \pm 0.017$. All isotope effects, and the $\beta_{ig}(V/K)$ value are in accord with the first chemical step being both the first irreversible and the rate-determining step in enzyme turnover, with a transition state in which there is little proton donation to the leaving group, the C–O bond is largely cleaved, there is significant nucleophilic participation, and the sugar ring is in a conformation derived from the ground-state 2C_5 chair. The apparent conflict between the $\beta_{ig}(V)$ value of -0.25 with all the kinetic-isotope-effect data can be resolved by the postulation of an interaction between the π system of the aglycone ring and an anionic or nucleophilic group on the enzyme.

INTRODUCTION

Neuraminidases (sialidases; EC 3.2.2.18) cleave sialic acid residues from oligosaccharides and the oligosaccharide moieties of glycoproteins and glycolipids. Biological functions associated with this *N*-acyl α -D-neuraminosyl-hydrolase activity include receptor recognition and masking, antigenic expression, and processes (including invasion) associated with the action of mammalian pathogens (Schauer, 1985).

A very great deal of structural information is available on influenza neuraminidase of various types, including the crystal structures of the uncomplexed enzyme (Varghese and Colman, 1991; Tulip et al., 1991) and that of the enzyme–*N*-acetylneuraminic acid complex (Varghese et al., 1992). This last is remarkable in that the sugar ring is bound in the $B_{2,5}$ conformation rather than the ground-state chair, and that the enzyme binds the α -anomeric form of the sugar, which is only 7.5% of the anomeric mixture at equilibrium (Friebolin et al., 1981).

The influenza enzyme works with retention of the anomeric configuration (Chong et al., 1992), but the crystal structure of the enzyme–*N*-acetylneuraminic acid complex reveals that there is no suitably placed protein side chain which could act as a nucleophile towards the anomeric centre, as is invariably found for retentive glycoside hydrolases without the substrate carboxylate (Sinnott, 1990). On the basis of site-directed-mutagenesis experiments, it was suggested that Glu-276, Glu-277 and His-274, all on the α -face of the sugar ring, were involved in proton transfer and electrostatic stabilization of an oxocarbenium ion intermediate (Lentz et al., 1987). The mechanistic studies of Chong et al. (1992) establish that the first chemical step in the hydrolysis of 4-methylumbelliferyl *N*-acetyl- α -D-neuraminic acid is subject to an intrinsic β -(D_2) deuterium kinetic-isotope effect of about 1.15, but that the second chemical step is

characterized by an intrinsic effect which is very small and probably slightly inverse. It was therefore proposed that the glycosyl-enzyme intermediate is an oxocarbenium ion. However, it could very well be the α -lactone; we have recently shown that, in the spontaneous hydrolysis of *p*-nitrophenyl *N*-acetyl- α -D-neuraminide, the C-1 carboxylate participates nucleophilically (Ashwell et al., 1992).

In contrast with the influenza neuraminidase, little structural information is available on the *Vibrio cholerae* enzyme, although crystals suitable for X-ray crystallography have been grown (Taylor et al., 1992) and the enzyme has been cloned and expressed in *Escherichia coli* (Vimr et al., 1988). The *V. cholerae* enzyme, though, has been the subject of extensive synthetic organic chemically based studies of substrate specificity (Zbiral et al., 1989; Schreiner et al., 1991) and inhibitor power (Wallimann and Vasella, 1990; Bernet et al., 1990; Czollner et al., 1990; Vasella and Wyler, 1991).

We now report a study of the *V. cholerae* enzyme, using three mechanistic probes applied to aryl *N*-acetylneuraminide substrates: β -deuterium kinetic-isotope effects, leaving-group ^{18}O kinetic-isotope effects, and the effect of leaving-group acidity on Michaelis–Menten parameters. These studies indicate that the *V. cholerae* enzyme has a very different mechanism to the influenza enzyme, in particular that the reactive sugar conformation is not the $B_{2,5}$ conformation seen in the influenza neuraminidase–*N*-acetylneuraminic acid complex.

On the basis of a report that the neuraminidases of *Clostridium perfringens* and *Arthrobacter ureafaciens* operated with retention of configuration (Friebolin et al., 1980), it has been widely assumed that all microbial sialidases act with retention. However, classification of glycosidases by hydrophobic-cluster analysis (Henrissat, 1991) puts influenza and *C. perfringens* enzymes in different classes, and it has been shown (Gebler et al., 1992) that the Henrissat classification does indeed

Abbreviation used: f.a.b., fast atom bombardment.

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correlate with the stereochemical course of the catalysed reaction. It therefore seemed worthwhile to examine briefly the stereochemical course of the *V. cholerae* enzyme.

METHODS AND MATERIALS

Labelled and unlabelled *N*-acetylneuraminides, except for 4-nitrophenyl 2- α -D-*N*-[18 O]acetylneuraminide, have been described previously (Ashwell et al., 1992). 4-Nitro-1-[18 O]phenol was made from 1-fluoro-4-nitrobenzene and $H_2^{18}O$ by the method of Winkel et al. (1989); fast-atom-bombardment (f.a.b.) m.s. revealed that two separate batches were 85 and 75% ^{18}O -labelled. The labelled 4-nitrophenol was converted into the labelled *N*-acetylneuraminide (m.p. 130–132 °C) as for the unlabelled compound (Ashwell et al., 1992): f.a.b.m.s. confirmed that label had not been washed out during the alkaline glycosidation reaction.

Affinity-chromatography-purified *Vibrio cholerae* neuraminidase, provided as a solution in 0.15 M NaCl/4 mM $CaCl_2$, was purchased from Sigma (St. Louis, MO, U.S.A.; batch nos. 121H-4008-1 and 39F-4041-2) and was used without further purification.

Kinetic measurements were made on a Perkin–Elmer Lambda 6 spectrophotometer fitted with an electrically (Peltier-effect) thermostatically controlled cell block. For determination of k_{cat} and K_m values, at least seven measurements of initial rate were made between $K_m/3$ and $3K_m$; reactions were initiated by addition of enzyme stock solution (10 μ l) to substrate (250–1000 μ l) and the absorbance was monitored continuously. Wavelengths and absorption coefficients differences (measured as the difference between the absorption coefficient of the glycoside and that of the phenol) are given in Table 1. Initial rates were fitted to a rectangular hyperbola using the non-linear least-squares program *Kaleidagraph* (Synergy, PCS Inc.). All rates were standardized on 4-nitrophenyl glycoside, and k_{cat} values were calculated on the assumption that enzyme of the highest activity was 100%-pure, fully active enzyme of molecular mass 82 kDa (Galen et al., 1992).

Kinetic-isotope effects on V_{max} were measured from direct comparison of zero-order rates with $[S] = 7K_m$ substrate, whereas effects on V_{max}/K_m were measured from direct comparison of first-order rate constants with $[S] = K_m/25$; first-order rate constants were calculated using *Kaleidagraph*. For both types of isotope effect, data were compared pairwise as obtained in the sequence light, heavy/heavy, light/light, heavy/etc.

Kinetic data pertain to 0.050 M sodium acetate buffer, pH 5.0, or 0.050 M sodium phosphate buffer, pH 8.0, at 37.0 °C. A

literature protocol for assay of *V. cholerae* neuraminidase (Potier et al., 1979) led us to assume that saturation of this Ca^{2+} -dependent enzyme could be maintained without additional Ca^{2+} in the assay buffer. The avoidance of excess Ca^{2+} seemed prudent, in the light of the well-known ability of carbohydrates to form complexes with Ca^{2+} and the established effect of this phenomenon on rates of non-enzymic hydrolysis of glycosides (Lönnerberg and Vesala, 1980). However, after most of the present work had been completed, the findings of Taylor et al. (1992) on the importance of added Ca^{2+} for structural integrity of the enzyme led us to verify this assumption. In fact, addition of enzyme stock solution to assay buffer containing 1.0 mM $CaCl_2$, at a concentration of 4-nitrophenyl *N*-acetyl- α -D-neuraminide substrate of K_m and $7K_m$, gave rates 2–3% lower than in the absence of added Ca^{2+} . Similarly, rates at $7K_m$ were strictly proportional to the amount of (Ca^{2+} -containing) enzyme stock solution added. We were therefore reassured that our kinetic measurements do indeed pertain to Ca^{2+} -saturated enzyme.

RESULTS AND DISCUSSION

In Figure 1 are displayed the time courses of u.v. absorbance and optical rotation as *p*-nitrophenyl *N*-acetyl- α -D-neuraminide (10 mM) reacts with neuraminidase. The cleavage of the glycone–aglycone bond is essentially complete after 3 h, but the rotation changes continue for a further 24 h. The mutarotation of *N*-acetylneuraminic acid is slow (it has a half-life of 80 min at p^H 5.4 and 30 °C; Friebohn et al., 1980), and the equilibrium lies 92.5% towards the β -anomer. If *V. cholerae* neuraminidase had reacted with inversion to give initially the β -anomer of *N*-acetylneuraminic acid, therefore, most of the rotation change would have been associated with cleavage of the glycone–aglycone bond, and there would have been little further change as 7.5% of the α -anomer was slowly produced by spontaneous mutarotation. It is clear, though, that less than one third of the total rotation change is associated with hydrolysis, and that there is a substantial subsequent change after hydrolysis is essentially complete. *V. cholerae* neuraminidase therefore does indeed catalyse hydrolysis with overall retention of configuration.

In Table 1 are set out Michaelis–Menten parameters for hydrolysis of seven aryl glycosides of the enzyme. Both $\log k_{cat}$ and $\log(k_{cat}/K_m)$ correlate well with the pK_a of the phenol leaving group, giving $\beta_{lg}(V)$ of -0.25 ($r = -0.95$) and $\beta_{lg}(V/K)$ of -0.73 ($r = -0.93$). The good correlation in both cases suggests that glycone–aglycone bond fission governs both k_{cat} and k_{cat}/K_m , although the difference in β_{lg} values suggests that hydrolysis of a glycosyl-enzyme intermediate may contribute to k_{cat} .

Table 1 Michaelis–Menten parameters for hydrolysis of aryl *N*-acetyl- α -D-neuraminides by *Vibrio cholerae* neuraminidase, at pH 5.0 and 37.0 °C

Parent phenol	Wavelength (nm)	$\Delta\epsilon$ ($cm^{-1} \cdot M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)
4-Nitrophenol	340	1755	107	1.63
	390	681		
3-Nitrophenol	340	594	63	1.93
4-Cyanophenol	262	532	62	1.97
3,4-Dichlorophenol	280	1130	51	1.91
3-Chlorophenol	280	781	48	11.0
4-Chlorophenol	280	1143	22.9	5.08
Phenol	270	837	21.6	41

Figure 1 Time courses of (a) absorbance at 390 nm in a 1 mm-path-length cell and (b) optical rotation at 546 nm in a 10 cm-path-length cell of a solution containing *p*-nitrophenyl *N*-acetyl α -D-neuraminide (5.0 mM) and *V. cholerae* neuraminidase (2.7×10^{-6} mM) in sodium acetate buffer, pH 5.0, at 37.0 °C

Table 2 Kinetic-isotope effects on the hydrolysis of *p*-nitrophenyl *N*-acetyl α -*D*-neuraminide by *Vibrio cholerae* neuraminidase at 37.0 °C

Quoted errors are standard deviations on eight pairwise comparisons, except where stated.

Site of substitution	(<i>V</i>) at pH 5.0	(<i>V</i> / <i>K</i>) at pH 5.0	(<i>V</i>) at pH 8.0
<i>proR</i> 3-[² H]	1.037 ± 0.014	1.030 ± 0.017	
<i>proS</i> 3-[² H]	1.018 ± 0.015	1.030 ± 0.017	
3-[² H] ₂	1.059 ± 0.010	1.058 ± 0.019	1.053 ± 0.010
2-[¹⁸ O]	1.040 ± 0.016*	1.046 ± 0.015 (<i>n</i> = 9)	

* The average of two separate determinations with different batches of labelled substrate, which gave effects of 1.035 ± 0.014 (*n* = 8) and 1.045 ± 0.013 (*n* = 8).

for the more acidic aglycones. It is quite clear, however, that non-covalent events, such as product release or conformation changes, do not mask the chemistry with this enzyme and these substrates. The large, negative value of $\beta_{18}(V/K)$, indicates that the change in charge at the aglycone oxygen atom between the glycoside in free solution and the first enzymic transition state is 73 % of that occurring on ionization of a phenol. This implies that there is little proton donation to the leaving group at this transition state.

Kinetic-isotope effects consequent upon isotopic substitution at various sites are given in Table 2. The leaving group ¹⁸O effects are particularly informative about which step is rate-limiting. The ¹⁸(*V*/*K*) value is very similar to those observed (for *p*-nitrophenyl glycosides) by Rosenberg and Kirsch (1981b) for sweet-almond (*Amygdalus communis*) β -glucosidase A (1.0377 ± 0.0061) and lysozyme (1.0467 ± 0.0015). These high values all indicate a late transition state and confirm that there is little proton or no proton donation to the leaving group, in accord, in the case of *V. cholerae* neuraminidase, with the $\beta_{18}(V/K)$ value. All hydrolyses of glycosides (such as the acid-catalysed hydrolyses of methyl glucopyranosides), where the leaving oxygen is completely protonated before C–O cleavage, are associated with leaving-group kinetic-isotope effects of 1.024–1.026 (Bennet and Sinnott, 1986).

The ¹⁸(*V*) value reported in Table 2 is the ratio of rates of increase in *A*₃₉₀, without correction for the isotope effect on the *pK*_a of *p*-nitrophenol (1.018; Rosenberg and Kirsch, 1981a). The justification for this lies in the absorption-coefficient difference in Table 1: at 390 nm at pH 5.0 this is 681 cm⁻¹·M⁻¹ for the *p*-nitrophenol glycoside. However, the absorption coefficient of *p*-nitrophenolate (measured in 1.5 M Na₂CO₃) is 1.54 × 10⁴ cm⁻¹·M⁻¹. Therefore, were the absorption coefficient change at 390 nm at pH 5.0 wholly due to *p*-nitrophenolate, it would be only 97 cm⁻¹·M⁻¹. It is clear that most of the absorption coefficient change is between the glycoside and un-ionized *p*-nitrophenol, which is not affected by the isotope effect on *pK*_a.

The ¹⁸(*V*) effect therefore indicates that glycone–aglycone bond cleavage determines *k*_{cat} for the most acidic aglycone used. The same conclusion comes from the identity, within experimental error, of (*V*) and (*V*/*K*) effects for the three types of secondary deuterium isotope effects measured. Further, the similarity of the largest isotope effect observed (that for dideutero substitution) at the optimum pH of 5.0 and the non-optimal pH of 8.0 further confirms that the effects measured at pH 5.0 are indeed intrinsic.

All four isotope effect measurements concur in indicating the same glycone–aglycone-cleavage step determines both *k*_{cat} and *k*_{cat}/*K*_m, yet the $\beta_{18}(V)$ and $\beta_{18}(V/K)$ values are radically different.

It therefore appears that there may be a polar effect on binding, operating on the aromatic aglycone. The effect is so large that it is unlikely to arise by interactions at the glycosidic centre; rather it is possible to envisage direct interactions of, say, a negatively charged group on the protein with the aromatic π system of the leaving group. Such an interaction would be more sensitive to the electronic nature of the aromatic ring than the *pK*_a of the phenol: rates of nucleophilic aromatic substitution, for example, obey the Hammett equation with ρ^- values of +4, compared with +2 for equilibrium ionization of substituted phenols (Isaacs, 1987). The possible nature of this interaction is discussed below in connection with transition state conformation and the pattern of inhibitory behaviour of sialic acid derivatives (Wallimann and Vasella, 1990; Bernet et al., 1990).

β -Deuterium kinetic isotope effects are particularly informative about transition-state geometry and charge development. They have their origin in two phenomena, hyperconjugation and the inductive effect of deuterium, and can be considered in terms of eqn. (1):

$$\ln(k_H/k_D) = \cos^2\theta \cdot \ln(k_H/k_D)_{\max} + \ln(k_H/k_D)_I \quad (1)$$

The first term represents hyperconjugation of the C–L σ orbital with an electron-deficient p-orbital on an adjacent carbon atom: θ is the dihedral angle between the C–L bond and this orbital. $\ln(k_H/k_D)_{\max}$ is the maximal hyperconjugative effect obtained when the C–L bond and the *p* orbital are exactly eclipsed, and increases as the positive charge on the adjacent carbon atom increases, with the associated weakening of the C–L bond. The second term represents a small, geometry-independent, inductive deuterium effect, resulting from the slightly electron-donating inductive effect of deuterium. The inductive effect is thus in the opposite sense to the hyperconjugative effect.

The inductive effect is, however, geometry-independent, so the relative magnitudes of the kinetic isotope effects of the individual diastereotopic β -hydrons enable qualitative information about the reactive conformation to be immediately obtained. Furthermore, an internal check on the measurements is available, since the measured effect from two deuteriums atoms should be the product of the individual single-deuterium effects. With *V. cholerae* neuraminidase (Table 2), the dideutero effects are indeed the product of the two single deuterium effects. Moreover, the effect from a *proR* deuterium is the same as, or somewhat greater than, the effect from a *proS* deuterium. This immediately rules out reaction from the B_{2,5} conformation (II), since in this conformation the C-3-*proS* hydron bond makes a dihedral angle of approx. 180° with the breaking C–OAr bond, whereas the C-3-*proR* hydron bond makes a dihedral angle of approx. 60° with the breaking C–OAr bond. Reaction from this conformation would thus result in the *proS* effect being bigger than the *proR*, and this is not observed.

It is noteworthy that the *proS* effect is indeed bigger than the *proR* for the first bond-breaking step of leech sialidase L, a transferase which yields 2,7-anhydro-*N*-acetylneuraminic acid (III) as product (Sinnott et al., 1993); in this case reaction is occurring from somewhere along the ³*B*₂ ⇌ ⁸*B*₂ ⇌ B_{2,5} portion of the skew-boat pseudorotational itinerary.

If indeed the complex of influenza neuraminidase with *N*-acetylneuraminic acid in the $B_{2,5}$ conformation does reflect the reactive conformation of true substrates, then the influenza and *V. cholerae* enzymes must have radically different mechanisms. The relative magnitudes of the effects for this enzyme are consistent with reaction through the ground-state 2C_5 chair (I), perhaps flattened towards the 4H_5 half-chair (which would result in the *proR* effect being somewhat bigger than the *proS* effect, as is observed for non-enzymic hydrolysis (Ashwell et al., 1992). A further difference is the much lower degree of charge development at C-2 in the first chemical transition state. The intrinsic deuterio effect for the *V. cholerae* enzyme (1.06) is much lower than that for the influenza enzyme (1.15; Chong et al., 1992) and is lower even than for spontaneous hydrolysis where the carboxylate participates nucleophilically (1.08; Ashwell et al., 1992).

We have therefore uncovered two unusual features of this enzyme: low charge development at the reaction centre, and a strong polar effect on the binding of aryl glycosides. A third unusual feature has been discovered by Vasella's group, that, with certain pairs of inhibitors epimeric at C-2 (e.g. IV; Wallimann and Vasella, 1990), the epimer homologous with substrate, with the carboxylic acid group axial, is bound the less tightly. Their detailed interpretation, in terms of a $B_{2,5}$ -like transition state in which the carboxylate group made favourable contacts with, say, a guanidino residue only at the transition state, is not compatible with our kinetic-isotope-effect measure-

ments, for reasons outlined above. However, their basic idea of an active site in which an axial orientation of the substrate carboxylic acid is somehow destabilized will rationalize our results if the destabilization occurs by repulsion of like charges, rather than attraction of opposite ones. If there were a carboxylate group on the enzyme placed approximately as shown in (V), repulsion between like charges would increase the nucleophilicity of the substrate carboxylate group, resulting in less charge development at C-2 at the transition state. The same carboxylate group might also participate in stabilizing interactions with electron-deficient π systems of aryl glycosides.

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REFERENCES

- Ashwell, M., Guo, X. and Sinnott, M. L. (1992) *J. Am. Chem. Soc.* **114**, 10158–10166
 Bennet, A. J. and Sinnott, M. L. (1986) *J. Am. Chem. Soc.* **108**, 7287–7294
 Bernet, B., Murty, A. R. C. B. and Vasella, A. (1990) *Helv. Chim. Acta* **73**, 940–958
 Chong, A. K. J., Pegg, M. S., Taylor, N. R. and von Itzstein, M. (1992) *Eur. J. Biochem.* **207**, 335–343
 Czollner, L., Kuszmann, L. and Vasella, A. (1990) *Helv. Chim. Acta* **73**, 1338–1358
 Friebohn, H., Supp, M., Brossmer, R., Keilich, G. and Ziegler, D. (1980) *Angew. Chem. Int. Ed. Engl.* **19**, 208–209
 Friebohn, H., Kunzelmann, P., Supp, M., Brossmer, R., Keilich, G. and Ziegler, D. (1981) *Tetrahedron Lett.* **22**, 1383–1386
 Galen, J. E., Ketley, J. M., Fasano, A., Richardson, S. H., Wasserman, S. S. and Kaper, J. B. (1992) *Infect. Immun.* **60**, 406–415
 Gebler, J., Gilkes, N. R., Claeysens, M., Wilson, D. B., Béguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller, R. C., Warren, R. A. J. and Withers, S. G. (1992) *J. Biol. Chem.* **267**, 12559–12561
 Henrissat, B. (1991) *Biochem. J.* **280**, 309–316
 Isaacs, N. S. (1987) *Physical Organic Chemistry*, pp. 453–458, Longman, Harlow
 Lentz, M. R., Webster, R. G. and Air, G. M. (1987) *Biochemistry* **26**, 5351–5358
 Lönnberg, H. and Vesala, A. (1980) *Carbohydr. Res.* **78**, 53–59
 Potier, M., Mameli, L., Bélière, M., Dallaire, L., and Melançon, S. B. (1979) *Anal. Biochem.* **94**, 287–296
 Rosenberg, S. and Kirsch, J. F. (1981a) *Biochemistry* **20**, 3189–3196
 Rosenberg, S. and Kirsch, J. F. (1981b) *Biochemistry* **20**, 3196–3204
 Schauer, R. (1985) *Trends Biochem. Sci.* **10**, 357–360
 Schreiner, E., Zbiral, E., Kleinedam, R. G. and Schauer, R. (1991) *J. Liebig's Ann. Chem.* **129**–134
 Sinnott, M. L. (1990) *Chem. Rev.* **90**, 1171–1202
 Sinnott, M. L., Guo, X., Li, S. C., Li, Y.-T. (1993) *J. Am. Chem. Soc.* **115**, 3334–3335
 Tulip, W. R., Varghese, J. N., Baker, A. T., van Donkelaar, A., Laver, W. G., Webster, R. G. and Colman, P. M. (1991) *J. Mol. Biol.* **221**, 487–497
 Taylor, G., Vimr, E., Garman, E. and Laver, G. (1992) *J. Mol. Biol.* **226**, 1287–1290
 Varghese, J. N. and Colman, P. M. (1991) *J. Mol. Biol.* **221**, 473–486
 Varghese, J. N., McKimm-Breschkin, J. L., Caldwell, B., Kortt, A. A. and Colman, P. M. (1992) *Proteins* **14**, 327–332
 Vasella, A. and Wyler, R. (1991) *Helv. Chim. Acta* **74**, 451–463
 Vimr, E., Lawrisuk, L., Galen, J. and Kaper, J. B. (1988) *J. Bacteriol.* **170**, 1495–1504
 Wallimann, K. and Vasella, A. (1990) *Helv. Chim. Acta* **73**, 1359–1372
 Winkel, C., Aarts, M. W. M., van der Heide, F. R., Buitenhuis, E. G., and Lugtenberg, J. (1989) *Recl. Trav. Chim. Pays-Bas* **108**, 139–146
 Zbiral, E., Schreiner, E., Salunkhe, M. M., Schulz, G., Kleinedam, R. G. and Schauer, R. (1989) *J. Liebig's Ann. Chem.* **519**–526