Effect of Aryl Substituents on the Kinetics of Inactivation of Glycosidases by Glycosylmethylaryltriazenes: Examination of the 'Suicide' Nature of these Inactivations

Michael L. Sinnott * and George T. Tzotzos
Department of Organic Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS
(in part) **Susan E. Marshall**
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TH

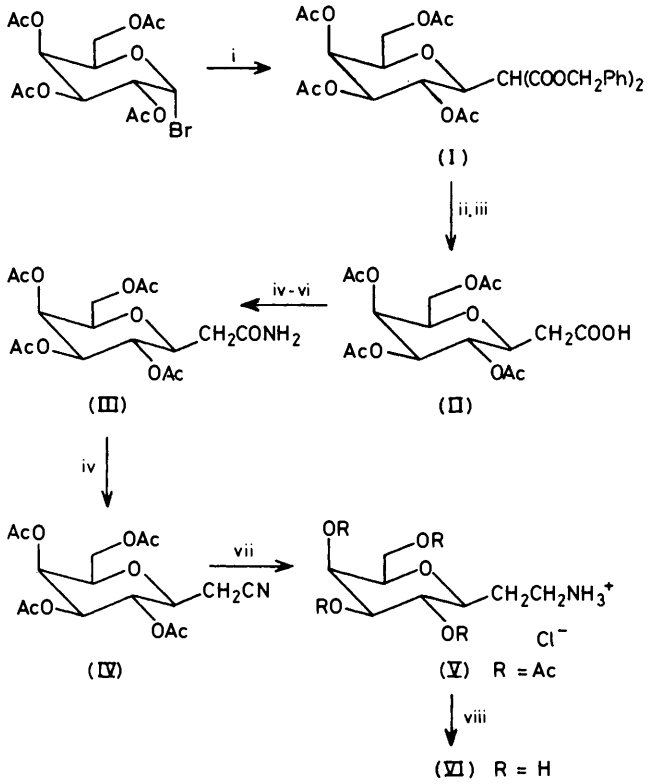
The inactivation of the Mg^{2+}-free form of the *lacZ*-β-galactosidase of *Escherichia coli* at 25.0 °C by various β-D-galactopyranosylmethylaryltriazenes resembles the spontaneous, rather than the acid-catalysed decomposition of alkylaryltriazenes in that both the maximum first-order rate constant, and the second-order rate constant, are governed by a negative β_{lg} value at pH 7.0 and at pH 8.0. Less extensive data with the β-xylosidase of *Penicillium wortmanni* and β-D-xylopyranosylmethylaryltriazenes give a similar result. Although the decomposition of the 2-(β-D-galactopyranosyl)ethyl compounds in aqueous solution is 5—10 fold faster than their lower homologues, β-galactosidase inactivation is 3—13 times slower. β-D-Galactopyranosylmethyl-*p*-nitrophenyltriazene does not inactivate the lectin, RCA ricin.

In the first paper of this group,[1] we showed that the equilibrium constant describing the proportion of unconjugated to conjugated tautomer in solutions of propylaryltriazenes is governed by the pK_a of the corresponding anilinium ion with a β value of −0.30. Alteration of the alkyl group has little effect. In the second paper,[2] we showed that the decomposition of alkylaryltriazenes in aqueous media can proceed by two processes, both leading to alkanediazonium ions. In the first, governed by positive β_{lg} values, a molecule of Brønsted acid is immobilised at the transition state, whose structure varies with the strength of the acid and the basicity of the aniline product in a way which can be rationalised by the use of a More O'Ferrall–Jencks diagram. The second process is a simple, spontaneous departure of anilide anion, governed by the pK_a of the departing aniline with a β_{lg} value of −0.25.

Glycosylmethylaryltriazenes have considerable potential as suicide substrates of glycosidases,[3] particularly as they work *in vivo*.[4] The major limitation on their use is their instability in aqueous media, but sufficient data has now been accumulated [2] to allow this instability to be minimised in a rational way for any given case. One of the variables in such a minimisation is the aryl group substituent, but this also in principle affects the enzymic suicide process. We now report an investigation of substituent effects on the most well understood of these suicide inactivations that of the Mg^{2+}-free form of the *lacZ*-β-galactosidase, of *Escherichia coli*,[3,5] and less extensive data on the β-xylosidase of *Penicillium wortmanni*.[3] Our results indicate that the enzyme inactivations resemble the spontaneous rather than the acid-catalysed decomposition of triazenes.

This surprising result caused us to seek further confirmation that these enzyme inactivations were indeed suicide processes. Our first test was to measure the rate of inactivation of β-galactosidase by 2-(β-D-galactopyranosyl)ethyltriazenes, in which the electrophilic centre is removed from the active site by an additional methylene group. The parent 2-(β-D-galactopyranosyl)ethylamine was synthesised using as a key step the condensation of the sodium salt of dibenzyl malonate with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide, analogously to a reaction reported in the *gluco* series [6] (Scheme 1).

The second test was to examine carefully the effect of β-D-galactopyranosylmethyl-*p*-nitrophenyltriazene on a protein which binds D-galactopyranosyl derivatives, but does not transform them. If the inactivation is of the suicide type, then such a protein would not be inactivated. The problem has already been addressed to some extent, in that ready inactivation of *lac*-repressor by the *galacto*-compound was not observed,[3] but the limited amount of protein available, and the cumbersome assay (equilibrium dialysis), made for rather high upper limits on the inactivation rate. We now report a similar study on a more readily available galactoside-binding protein, the non-toxic, 120 000 MW component of the castor oil bean lectin, RCA, by a more sensitive technique, fluorescence polarisation. RCA contains two equivalent galactopyranose binding sites, and has some preference for β-derivatives.[7]



Scheme 1. *Reagents*: i, $CH_2(COOCH_2Ph)_2$–NaH–$(CH_2OCH_3)_2$; Pd–H_2; iii, heat; iv, $SOCl_2$; v, NH_3–Et_2O; vi, Ac_2O–pyridine; vii, H_2–Pd–C–EtOH–$CHCl_3$; viii, MeOH–HCl

The binding of the fluorescent ligand 4-methylumbelliferyl β-D-galactopyranoside to RCA has been studied by both fluorescence polarisation and equilibrium dialysis;[8] the dissociation constant of the complex is 42 μmol l^{-1} at 25 °C and pH 7.2.

The polarisation p of fluorescent emission is defined in terms of the fluorescent light intensities polarised either parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to the electric vector of the exciting polarised radiation [equation (1)]. Thermal tumbling

$$p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad (1)$$

motion decreases p, but if the fluorophore is bound to a macromolecule this decreases; p is a linear function of the concentration of protein–fluorophore complex. In practice, for small changes in p, $I_{\parallel}/I_{\perp}$ is also a linear function of the concentration of protein–fluorophore complex.[9]

Experimental

Dibenzyl C-(2,3,4,6-*Tetra*-O-*acetyl*-β-D-*galactopyranosyl*)-*malonate* (I).—Dibenzyl malonate (45 ml, 0.18 mol) was added dropwise, with stirring, to a suspension of pentane-washed sodium hydride (3.73 g net, 0.16 mol) in $LiAlH_4$-dried 1,2-dimethoxyethane (130 ml), under an atmosphere of dry nitrogen. The clear solution was allowed to cool to room temperature, and then 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (20 g, 0.049 mol) in the minimum volume of $LiAlH_4$-dried 1,2-dimethoxyethane was added with stirring. The whole was allowed to stand for 48 h, and was then concentrated at below 30 °C to *ca.* 75 ml. Ether (500 ml) was then added, and the ethereal solution was washed with 1M aqueous HCl (100 ml), and then with water until the washings were colourless, dried ($MgSO_4$) and evaporated. Dibenzyl malonate was removed by trituration with pentane, the pentane was evaporated off, and the syrup taken up in warm ethanol. The resultant crystals were recrystallised twice from ethanol–ether to give the *product* (5.8 g, 19%), m.p. 102—104 °C, $[\alpha]_D^{25}$ −13.7° (*c* 1 in $CHCl_3$), ν (CH) 3 030, δ (CH) 1 600, and ν (C=O) 1 740 cm^{-1} (Nujol) (Found: C, 60.45; H, 5.65. $C_{31}H_{34}O_{13}$ requires C, 60.6; H, 5.55%).

2,3,4,6-*Tetra*-O-*acetyl*-β-D-*galactopyranosylacetic Acid Hydrate* (II).—Diester (I) (1.0 g) was taken up in a small volume of ethanol and added to a suspension of 20% palladium on charcoal[10] (0.3 g) in ethanol. The mixture was hydrogenated at room temperature in a Parr hydrogenator (initial pressure 3.5 atm.) until there was no further uptake of hydrogen. The catalyst was then filtered off, and the ethanol was evaporated. The resultant syrup which t.l.c. [benzene–methanol–acetic acid (45 : 8 : 8 v/v) on silica gel] showed to contain two components, neither identical with diester (I) was taken up in glacial acetic acid, and heated for 3 h under reflux. The acetic acid was evaporated, and crystals appeared on addition of a few drops of water. Two recrystallisations from aqueous ethanol gave the *product* (0.41 g, 73%), m.p. 92—93 °C, $[\alpha]_D^{25}$ +12.8° (*c* 1.15 in $CHCl_3$), ν (OH) 3 470, 3 360 (H_2O), 2 800—2 500 (COOH), and ν (C=O) 1 740 cm^{-1} (Nujol) (Found: C, 46.9; H, 5.8. $C_{16}H_{22}O_{11}\cdot H_2O$ requires C, 47.05; H, 5.9%). The mother liquor from which the diester (I) crystallised was concentrated, hydrogenolysed, and decarboxylated under the same conditions. After the decarboxylation step, the tarry residue was triturated with a mixture of water and carbon tetrachloride. The aqueous layer, on concentration, gave crystals which after three recrystallisations from aqueous ethanol gave a further crop (1.1 g) of *product*, m.p. and mixed m.p. 90—92 °C.

C-(2,3,4,6-*Tetra*-O-*acetyl*-β-D-*galactopyranosyl*)*acetamide* (III).—Acid (II) (1 g) was dissolved in thionyl chloride (10 ml) and heated gently under reflux for 1—2 h. The excess thionyl chloride was evaporated off under reduced pressure, and ether (200 ml) through which ammonia gas had been bubbled, was added. The precipitate was filtered off, dissolved in a mixture of pyridine and acetic acid (1 : 2 v/v, 30 ml), and allowed to stand overnight. The solution was then poured into ice–water (100 ml), concentrated to 3.5 ml, and extracted with chloroform (20 ml). The chloroform was washed successively with an equal volume of saturated $NaHCO_3$ and 2N-HCl, and water (40 ml), and evaporated. Crystals appeared on addition of ether, and amide (III) (0.69 g, 69%) was obtained after four further recrystallisations from chloroform–ether, m.p. 166—167 °C, $[\alpha]_D^{25}$ +10.0° (*c* 1 in $CHCl_3$), ν (NH_2) 3 470, 3 360, amide ν (C=O) 1 680, 1 610, and ester ν (C=O) 1 735 cm^{-1} (Nujol) (Found: C, 49.15; H, 5.95; N, 3.35. $C_{16}H_{23}NO_{10}$ requires C, 49.35; H, 5.9; N, 3.6%).

(2,3,4,6-*Tetra*-O-*acetyl*-β-D-*galactopyranosyl*)*acetonitrile* (IV).—Lithium aluminium hydride failed to give clean production of amine from amide (III) so it was first dehydrated to the nitrile and then reduced catalytically. Amide (III) (1 g) was heated under reflux with thionyl chloride for 3 h, after which the excess thionyl chloride was distilled off, the residue was taken up in ether, and the ether was evaporated. The residue, which crystallised, was recrystallised twice from ethanol–ether to give *nitrile* (IV) (0.76 g, 80%), m.p. 130—131 °C, $[\alpha]_D^{25}$ +14.8° (*c* 0.8 in $CHCl_3$), ν (CN) 2 250 and ν(C=O) 1 740 cm^{-1} (Nujol) (Found: C, 51.6; H, 5.95; N, 3.8. $C_{16}H_{21}NO_9$ requires C, 51.75; H, 5.65; N, 3.75%).

2-(2,3,4,6-*Tetra*-O-*acetyl*-β-D-*galactopyranosyl*)*ethylamine Hydrochloride* (V).—Nitrile (IV) (0.74 g) was dissolved in ethanol (150 ml) containing chloroform (1—2 ml) and was reduced catalytically with hydrogen in a Parr hydrogenator over a 20% palladium–charcoal[7] catalyst (200 mg) for 6 h. The catalyst was filtered off, the solution was evaporated, and the residue was partitioned between chloroform (20 ml) and water (10 ml). Evaporation of the water gave a syrup which crystallised on addition of ether to give the *product* (0.56 g, 68%), m.p. 193—195 °C, $[\alpha]_D^{25}$ +12.3° (*c* 0.9 in water), ν (NH) 2 800—2 500 and ν(C=O) 1 740 cm^{-1} (Nujol) (Found: C, 46.4; H, 6.1; N, 3.6; Cl, 8.4. $C_{16}H_{26}ClNO_9$ requires C, 46.65; H, 6.3; N, 3.4; Cl, 8.65%).

2-(β-D-*Galactopyranosyl*)*ethylamine Hydrochloride* (VI).—De-*O*-acetylation of compound (V) was achieved in 95% yield with methanolic HCl;[11] the product was a very hydroscopic solid (Found: C, 39.3; H, 7.7; N, 5.7; Cl, 14.9. $C_8H_{18}ClNO_5$ requires C, 39.45; H, 7.4; N, 5.75; Cl, 14.55%).

N-*Benzoyl*-β-D-*galactopyranosylmethylamine* (VII) *and* N-*Benzoyl*-2-(β-D-*galactopyranose*)*ethylamine* (VIII).—These were obtained, in 82 and 72% yield, respectively, by benzoylation of the parent amines with *p*-nitrophenyl benzoate.[12] *Amide* (VII) had m.p. 165—167 °C, $[\alpha]_D^{25}$ −4.4° (*c* 0.45 in water) (Found: C, 56.7; H, 6.3; N, 4.75. $C_{14}H_{19}NO_6$ requires C, 56.55; H, 6.4; N, 4.7%). Amide (VIII) had m.p. 181—183 °C (Found: C, 57.6; H, 6.7; N, 4.3. $C_{15}H_{21}NO_6$ requires C, 57.9; H, 6.75; N, 4.5%).

N-*Benzyl*-β-D-*galactopyranosylcarboxamide* (N-*Benzyl*-2,6-*anhydro*-D-*glycero*-L-manno-*heptitonamide*).—Methyl 3,4,5,7-tetra-*O*-acetyl-D-glycero-L-*manno*-heptitonate[13] (0.5 g) was dissolved with gentle heating in benzylamine (1.35 ml, 10 equiv.), and the mixture was left to stand for 40 h at room temperature. Dry ether (50 ml) was added, the product was

filtered off and washed repeatedly with dichloromethane and ether, yield 0.32 g (88%), m.p. 218—220 °C, $[\alpha]_D^{25}$ +52° (*c* 1.4 in water) (Found: C, 56.35; H, 6.45; N, 4.65. $C_{14}H_{17}NO_6$ requires C, 56.55; H, 6.4; N, 4.7%).

β-D-Galactopyranosylmethyl-, β-D-Xylopyranosylmethyl-, and 2-β-D-Galactopyranosyl)ethyl-aryltriazenes.—These were prepared as described for the *p*-nitrophenyl compounds,[3,5] except that 3,5-dichlorobenzenediazonium tetrafluoroborate was so insoluble in water that it was necessary to add 20% (v/v) of methanol as co-solvent. The carbohydrate triazenes were characterised by the conformity of their u.v. spectra to the well characterised propyl compounds. The *p*-cyanophenyl compounds had a broad maximum at 295—310 nm and an isosbestic point during decomposition at 278 nm; the 3,4- and 3,5-dichlorophenyl-, and *p*-chlorophenyl-triazenes all had maxima at 280 nm. The free amine was liberated from 2-(β-D-galactopyranosyl)ethylamine hydrochloride by addition of the calculated volume of 0.1N-NaOH.

Kinetics of Inactivation of β-Galactosidase and β-Xylosidase.—*lacZ*-β-Galactosidase was isolated[5] from strain A324-5 of *E. coli* K12, constitutive for, and merodiploid with respect to, the *lac*-operon.[14] Mg^{2+} ions were removed by dialysis against EDTA,[15] and inactivations were carried on at 25.0 °C in 0.1M-sodium EDTA buffer, pH 7.0 or 8.0. Portions of an inactivation solution containing enzyme and a given concentration of triazene were assayed at 25.0 °C against 0.3mM-*p*-nitrophenyl β-D-galactopyranoside in 0.1M-sodium phosphate buffer, pH 7.0, 1.0mM in $MgCl_2$. Increase in absorbance at 400 nm was monitored in a Unicam SP 1800 spectrophotometer fitted with a thermostatted cell block.

The equilibrium mixture of γ- and δ-lactones produced by dissolution of D-galactono-γ-lactone in buffer was used as a protecting agent. The apparent K_i value for binding to Mg^{2+}-free enzyme at pH 7.0 was 1.0 mmol l^{-1} and at pH 8.0 7.3 mmol l^{-1}, measured from initial rates at each of between four and six inhibitor and *p*-nitrophenyl galactoside concentrations.

β-Xylosidase from *Penicillium wortmanni* was inactivated and assayed as described previously.[3]

First-order rate constants (k_{obs}) for enzyme inactivation at each individual triazene concentration were obtained by least squares treatment of plots of log (activity) against time; original data are available in Supplementary Publication No. SUP 23423 (18 pp.).* Maximal rates of inactivation, and dissociation constants for the non-covalent enzyme–triazene complex, were obtained by linear least squares treatment of plots of k_{obs} against k_{obs}/[Triazene] (Eadie plot). Triazene concentrations were estimated from the u.v. absorbance at the appropriate wavelength, assuming extinction coefficients identical to those of these crystalline propyl compounds. Since alkyl substituents do not affect the position of tautomeric equilibrium in triazenes,[1] this procedure is valid. Spontaneous decomposition of triazenes was monitored in a Unicam SP 8-200 spectrophotometer fitted with the manufacturer's thermostatting system; first-order rate constants were calculated by the Guggenheim procedure. Semilogarithmic plots of activity against time form part of SUP 23423, as do Lineweaver–Burk plots for inhibition by galactonolactone.

Attempted Inactivation of RCA Ricin.—*Ricinus communis* agglutinin, molecular weight 120 000, was purchased from Boehringer Mannheim Ltd. 4-Methylumbelliferyl β-D-galactopyranoside, m.p. 139—140°, was synthesised by standard procedures, and contained no detectable amounts of free aglycone, as established by the invariance of its fluorescence during pH-titration. Triazene inactivations were carried out in the buffer used throughout these experiments (0.15M-sodium phosphate, pH 7.0, 0.1M in sodium chloride) at room temperature for 48 h. Before assay the inactivation solutions were dialysed for 20 h against 1 000 volumes of buffer, and then three further times against 200 volumes of buffer. Fluorescence polarisation measurements were made in apparatus constructed in the Department of Biochemistry, with excitation at 310 nm and monitoring at 370 nm. The fluorescence polarisation of ligand in the absence of protein was checked on an SLM Instruments Inc. photon counting spectrofluorimeter.

Excitation light is conducted from the light source (XBO 200 W Xe arc; Osram G.m.b.H., Berlin) through a double grating monochromator to the excitation polarizer and then to the quartz fluorescence cuvette. The emission filters are Barr and Stroud graded spectrum filters type UMS1. The contents of the cuvette were stirred constantly, allowing addition of reagents with a microsyringe without optical or electrical perturbation of the measurement system. The data were stored in one channel of a dual-channel recycling digital store (Transidyne General Corp., Ann Arbor) as 1 024 10-bit words. After the experiment, signals from the store are reconverted into voltages every 20 ms and displayed on an oscilloscope. The fluorescence cuvette was supported by properly thermostatted blocks inside the fluorimeter. All experiments were performed at (25 ± 0.2) °C.

Results and Discussion

(A) *Substituent Effects on Inactivation by Galactosylmethyl-aryltriazenes.*—For truly active-site directed irreversible inhibition, according to equation (1), first-order loss of enzymic

$$E + Tr \overset{K}{\rightleftharpoons} E{\cdot}Tr \xrightarrow{k_{max}} ETr^* \qquad (1)$$

activity should be observed, with a rate constant k_{obs}, where equation (2) holds and I is a competitive inhibitor of catalysis, of dissociation constant K_i. Because of the lability of the

$$k_{obs} = \frac{k_{max.}[Tr]}{[Tr] + K(1 + [I]/K_i)} \qquad (2)$$

triazenes, particularly those with electron-donating substituents, plots of log (activity) against time are linear only for the initial period of the inactivation. Kinetic parameters derived from these protons of the inactivation time course are given in Table 1; protection by D-galactonolactone was observed in all cases, It is quite clear that at both pH 7.0 and 8.0 electron-withdrawing substituents in the aryl group speed up the inactivation, *i.e.* β_{lg} is negative. More quantitative treatment of the data requires resolution of a number of ambiguities.

(i) $k_{max.}$ refers to the rate of enzyme inactivation, not to rate of decomposition of the triazene in the enzyme active site. In the case of the *p*-nitrophenyl compound, it is known that for every enzyme active site alkylated, four molecules of triazene are, on average, decomposed,[5] *i.e.* the kinetic scheme really should be (3). Because of the high rates of spontaneous

$$E + \beta Gal p{-}CH_2{-}N_3HAr \rightleftharpoons E{\cdot}\beta Gal pCH{-}N_2HAr \longrightarrow E{\cdot}\beta Gal pCH_2N_2^+{\cdot}NH_2Ar$$
$$E{\cdot}\beta Gal pCH_2N_2^+ \qquad E + \beta Gal pCH_2OH + NH_2Ar \qquad (3)$$

* For details, see Notice to Authors No. 7 in *J. Chem. Soc., Perkin Trans. 2*, 1981, Index Issue.

Table 1. Kinetics of inactivation of Mg^{2+}-free *lacZ*-β-galactosidase at 25.0 °C by β-D-galactapyranosylmethyl-X derivatives in 0.1M-sodium EDTA buffers

X	pH	$10^4 k_{max.}/s^{-1}$	K/mmol l^{-1}	$10^4 k_0/s^{-1}$ [a]
N_3H-*p*-$C_6H_4NO_2$	7.0	70 [b]	0.30 [b]	0.48 ± 0.002 [c]
N_3H-*p*-$C_6H_4NO_2$	8.08	13.4 ± 1.2 [d]	0.32 ± 0.05 [d]	0.26 ± 0.01 (at pH 8.25)
N_3H-*p*-C_6H_4CN	7.0	6.35 ± 0.11	0.42 ± 0.16	1.53
N_3H-*p*-C_6H_4CN	8.0	5.54 ± 0.16	1.6 ± 0.7	0.20
N_3H-3,5-$C_6H_3Cl_2$	7.0	4.29 ± 0.11	0.103 ± 0.006	2.64
N_3H-3,5-$C_6H_3Cl_2$	8.0	4.26 ± 0.23	0.55 ± 0.05	0.268
N_3H-3,4-$C_6H_3Cl_2$	7.0	4.3 ± 0.2	0.20 ± 0.03	4.52
N_3H-3,4-$C_6H_3Cl_2$	8.0	4.5 ± 0.3	0.91 ± 0.10	0.526
N_3H-*p*-C_6H_4Cl	7.0			26.7
N_3H-*p*-C_6H_4Cl	8.0	3.4 ± 0.3	1.6 ± 0.2	2.71
CH_2N_3H-*p*-$C_6H_4NO_2$	7.0	5.2 ± 0.5	0.17 ± 0.04	8.08
CH_2N_3H-*p*-$C_6H_4NO_2$	8.0	5.5 ± 1.2	1.07 ± 0.35	3.82
CH_2N_3H-3,5-$C_6H_3Cl_2$	7.0			10.3
CH_2N_3H-3,5-$C_6H_3Cl_2$	8.0	1.6 ± 0.2	1.6 ± 0.3	1.31

[a] Rate constant for decomposition of triazene. [b] Ref. 5. [c] P. J. Smith, unpublished data. [d] Ref. 3.

Table 2. Results of correlations of kinetic parameters for inactivation of Mg^{2+}-free *lacZ*-β-galactosidase by galactosylmethylaryltriazenes

pH	y	x	β_{lg}	r
7.0	log $k_{max.}$	pK_a ($ArNH_2$)	−0.18 ± 0.05	−0.92
7.0	log $k_{max.}$	pK_a ($ArNH_3^+$)	−0.42 ± 0.28	−0.73
8.0	log $k_{max.}$	pK_a ($ArNH_2$)	−0.014 ± 0.029	−0.27
8.0	log $k_{max.}$	pK_a ($ArNH_3^+$)	+0.006 ± 0.072	0.05
7.0	log $k_{max.}/K$	pK_a ($ArNH_2$)	−0.13 ± 0.10	−0.68
7.0	log $k_{max.}/K$	pK_a ($ArNH_3^+$)	−0.23 ± 0.34	−0.49
8.0	log $k_{max.}/K$	pK_a ($ArNH_2$)	−0.08 ± 0.06	−0.62
8.0	log $k_{max.}/K$	pK_a ($ArNH_3^+$)	−0.12 ± 0.15	−0.4

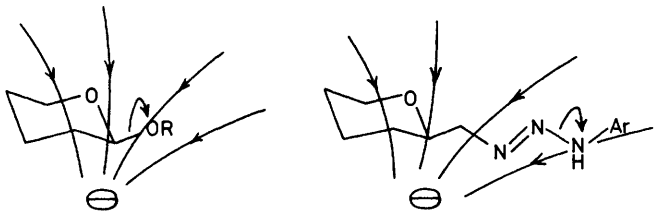
decomposition of galactosylmethylaryltriazenes with electron-donating substituents, which approach $k_{max.}$ values, it is not possible to measure capture efficiency directly, since concentrations of enzyme comparable to K would be required. However, since anilines are not bound specifically to β-galactosidase it is reasonable to assume that discrimination between the two pathways which are open to the system immediately after enzyme-catalysed triazene decomposition occurs after departure of aniline. These pathways are alkylation of enzyme or capture by water or buffer components. The assumption therefore is that capture efficiency is independent of arene substituent.

(ii) Only the unconjugated tautomer of the triazene will lead to galactosylmethanediazonium ion, but it is probable that the enzyme binds both tautomers: the situation is formally analogous to non-productive binding of substrate in enzyme catalysis. This will not affect correlations based on $k_{max.}/K$, but could affect those based on $k_{max.}$ In both types of correlation (Table 2) rates are corrected for the fraction of reactive tautomer, using data reported in the first paper of this group. This problem would not arise were the enzyme to catalyse triazene tautomerisation. However, since the reactive tautomer is the predominant one, such a process would not contribute significantly to the enzyme-catalysed triazene decomposition.

(iii) The pH-dependence of inactivation by the *p*-nitrophenyl compound is sigmoid, protonation of a group of pK_a 6.9 in the free enzyme, and 7.9 in the E·Tr complex being necessary for inactivation.[3] The similarity in maximal rates of inactivation at pH 7 and 8 for the *p*-cyano-, 3,4-dichloro-, and 3,5-dichloro-phenyltriazenes (Table 1) means that for these compounds the ionisation of this group in the E·Tr complexes occur at pH values >8, *i.e.* the perturbation of the pK_a is greater, as is reasonable, with the more hydrophobic ligands. This differential perturbation of a pK_a does not affect conclusions from $k_{max.}/K$ data (since this parameter is affected only by ionisations of free enzyme),[16] but does mean that only the correlation of log $k_{max.}$ at pH 7 is really meaningful.

The results of attempted structure–reactivity correlations (Table 2) indicate that only the correlation of log $k_{max.}$ against the pK_a of $ArNH_2$ at pH 7.0 is defensible. The β_{lg} value is, within the error limits, identical to that for spontaneous hydrolysis of propylaryltriazenes. There is little evidence of a correlation with the pK_a of $ArNH_3^+$. Correlations of log $k_{max.}/K$ are subject to considerable chemical noise, arising in all probability through adventitious steric interactions of substituents and the protein causing random variations in K. However, it is clear that log $k_{max.}/K$ correlates better with the pK_a of the aniline, rather than the anilinium ion, and exhibits, above the noise, a β_{lg} value which is negative.

To our considerable surprise, then, we are constrained to formulate the active-site process as resembling the spontaneous, rather than the acid-catalysed, hydrolysis of triazenes. There is room in the data for the presence of a minor amount of acidic assistance to N–N cleavage, and something must cause the faster rates of N–N bond fission in the E·Tr complex than in free solution, but any such assistance must be of a very partial, hydrogen-bonding nature. The binding of phenyl 1-thio-β-D-galactopyranosides to β-galactosidase is not significantly affected by the electronic effects of aryl substituents,[17] so it is likely that systematic effects on K in the present study are likewise insignificant. Since correlations of log(k_{cat}/K_m), unlike correlations of log $k_{max.}$, are unaffected by competitive inhibition by the ' wrong ' (conjugated) tautomer of the triazene, it is possible to interpret the β_{lg} values from correlation of these second-order rate constants, which are slightly higher than that for spontaneous hydrolysis of propyltriazenes, as indicating a very weak acidic assistance to N–N cleavage.



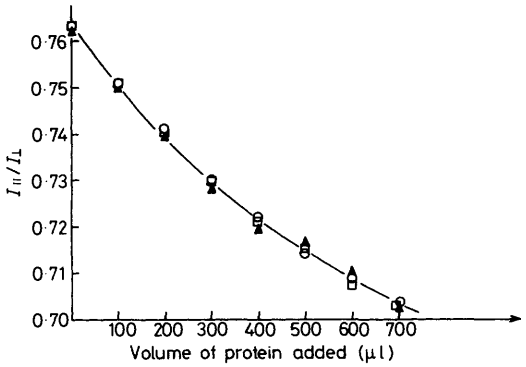
(1) (2)

(B) *Effect of Chain Extension. Inactivation by* 2-(β-D-*Galactopyranosyl)ethyltriazenes.*—The negative β_{lg} values obtained for enzyme inactivation with galactosylmethylaryltriazenes could be thought to indicate that these compounds are not, after all, suicide inactivators. It could be argued that decomposition of an alkylaryltriazene is slightly enhanced in the vicinity of a protein surface, without the special properties of an enzyme active site being required. Since it is of the utmost importance for the biological application of these reagents [4] for us to be confident that enzyme inactivation is indeed of a suicidal, rather than a merely accidental, nature, we addressed ourselves further to this question. If enzyme inactivation arises merely from the misadventure that an alkylaryltriazene adsorbed at a protein surface is up to 10^2 more labile than in free solution, rather than from a suicide process at the enzyme active site, then increasing the distance between the galactosyl residue and the triazene function by inserting a methylene group should not appreciably affect inactivation. Indeed, since the spontaneous non-enzymic reaction is faster because of the greater remoteness of the electron-withdrawing glycosyl residue, inactivation, on the ' misadventure ' model, could even be faster.

The data in Table 1 indicate that the galactosyl ethyl compounds are 3–13-fold worse inactivators of the enzyme, despite a 5—10-fold faster rate of non-enzymic decomposition. The presence of the triazene function in the enzyme active site must therefore be a requirement for enzyme inactivation.

(C) *Inactivation of the* β-*Xylosidase of* Pencillium wortmanni *by Xylosylmethylaryltriazene.*—The data in the previous two sections show clearly that, whilst triazene decomposition in the active site of the *lacZ*-β-galactosidase of *E. coli* is undoubtedly catalysed, it resembles the spontaneous, rather than the acid-catalysed, decomposition of alkylaryltriazenes in free solution. To establish that this was not merely a pecularity of the particular enzyme chosen, we briefly investigated substituent effects in activations of the β-xylosidase of *Pencillium wortmanni* by β-D-xylopyranosylmethylaryltriazenes. The *p*-nitrophenyl compound is so effective that saturation of enzyme by reagent cannot be observed, and only a bimolecular rate constant for inactivation ($k_{max.}/K$) is obtainable: 38 ± 6 l mol^{-1} s^{-1} at 25.0 °C in 0.1M-sodium EDTA buffer, pH 7.0.[3] Under the same conditions *p*-cyanophenyl compound inactivates the enzyme with $k_{max.}$ 8.1 ± 0.3 × 10^{-4} s^{-1}, k 36 ± 7 μmol l^{-1}, and the 3,5-dichlorocompound with $k_{max.}$ *ca.* 10^{-4} s^{-1}, K *ca.* 10^{-4} mol l^{-1}. Although the data does not warrant detailed interpretation, β_{lg} is again negative: the results with *lacZ*-β-galactosidase are not unique to that enzyme.

An alternative to the formulation of the suicide process as involving sufficient acid catalysis to accelerate the reaction, but insufficient to give a positive β_{lg} value comes from the theoretical work of Warshel [18] on the glycosidase lysozyme. He proposed that the active site of this enzyme was so constructed as to provide a ' supersolvent ': the electrostatic fields from the active site residues were so aligned as to promote the generation of an oxocarbonium ion. It is not unreasonable that a field evolved to promote process (1) should also promote to some extent process (2), although as a model this is unpleasingly mystical. Little is known about the active site of *P. wortmanni* β-xylosidase, but Met-500 [5] has been identified as being in the active site of β-galactosidase. Further, the presence of a counterionic carboxylate group, disposed in the same way as Asp-52 in lysozyme, is made likely by the discovery of Legler and Herrschen [19] that conduritol C epoxide is an active-site-directed irreversible inhibitor of β-galactosidase.

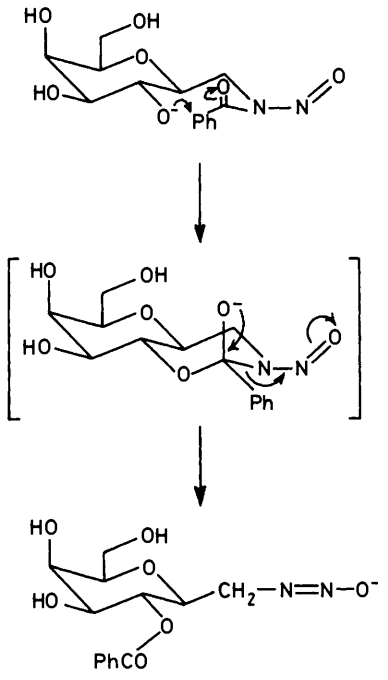


Variation of $I_{\parallel}/I_{\perp}$ of free 4-methylumbelliferyl β-D-galactopyranoside (1 μM) on addition of 100 μl portions of RCA: ▲, RCA (triazene free); ○, RCA (31 μM) incubated with triazene (4.6 mM) for 48 h at 22 °C; □, RCA (31 μM) incubated with triazene (1.14 mM) for 48 h at 22 °C

(D) *Attempted Inactivation of the Lectin, RCA Ricin.*—The Figure shows the effect on $I_{\parallel}/I_{\perp}$ of adding various volumes of lectin, which had and had not been exposed to triazene, to 1μM-4-methylumbelliferyl galactoside (2.5 ml). The stock solutions of the lectin were adjusted to 8.1μM using the extinction coefficient value of van Wauwe *et al.*[20] The fluorescence polarisation exhibited by the ligand in the absence of protein ($I_{\parallel}/I_{\perp}$ 0.763) was observed on two separate occasions, with different, clear solutions, and a value of 0.74 was measured on another apparatus. A value of p of *ca.* 0.07 for free ligand was reported, but not commented on, by Khan *et al.*[8] The rotation of a small molecule should be fast enough for the fluorescence to be completely depolarised. One possible explanation of the anomaly is that the ligand, which is only very sparingly soluble in water, aggregated in solution. For our purposes, it is clear that the protein binds to the fluorescent galactoside and that no loss of lectin active sites has occurred on incubation with triazene; we estimate a maximum rate of inactivation of 3 × 10^{-6} s^{-1}.

That the experimental system does indeed monitor fluorophore–protein complexation was confirmed by displacing protein-bound 4-methylumbelliferyl galactoside with increasing concentrations of lactose. A dissociation constant of 28 μmol l^{-1} for lactose was calculated (lit.,[21] 37 μmol l^{-1}).

(E) *Comments on the Synthesis of* 2-(β-D-*Galactopyranosyl)-ethylamine and Attempts to form* N-*Nitrosamides therefrom.*—The synthesis of compound (VI) was achieved by standard procedures, the two slightly unusual features being the partial decarboxylation of *C*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)malonic acid during catalytic hydrogenolysis of its dibenzyl ester, and the use of chloroform as a source of hydrogen chloride during the catalytic hydrogenation of nitrile (IV). The anomeric configuration of the C–glycosyl linkage was established in two ways. Brominative decarboxylation of acid (II) by a modified Hunsdiecker reaction,[22] and reaction of the



Scheme 2

syrupy product with anhydrous sodium acetate in dimethylformamide,[6] gave a 20% yield of a compound, m.p. 54—55 °C, presumed to be penta-*O*-acetyl β-D-galactopyranosylmethanol, m.p. 55—57 °C.[23] More direct evidence comes from the 200 MHz, ^{1}H n.m.r. spectrum of compound (V) in D_2O (measured on a JEOL FX 200 instrument with sodium 4,4-dimethyl-4-silapentanesulphonate as internal standard). A complex multiplet at δ 1.62—1.95 was assigned to the diastereotopic protons on C-2 since this carbon is the only main-chain carbon not bearing an electronegative substituent. Irradiation at δ 1.80 caused 1 H triplet of doublets centred on δ 3.86 to collapse to a doublet (splitting 10 Hz) and a 2 H triplet at δ 3.18 to collapse to a singlet. The signal at δ 3.18 is therefore due to the methylene group at C-1 and that at δ 3.86 due to the anomeric proton. The splitting of the latter establishes the β-configuration of the galactosyl moiety. Complete spectra are available in SUP 23423.

2-(β-D-Galactopyranosyl)ethylamine was synthesised orginally to explore the possibilities of using the nitrosamide deamination route [24] to affinity-label glycosyl-binding proteins. We had found that *N*-benzoyl-*N*-nitroso-β-D-galactopyranosylmethylamine decomposed within the time of addition to aqueous solutions of neutral pH, and we reasoned that this was probably due to effective neighbouring group participation by the 2-OH of the galactosyl moiety (Scheme 2).[5] Were this the case, a structure in which such participation would require formation of a seven-membered ring should be more stable. In the event, even though *N*-benzoyl-2-(β-D-galactopyranosyl)ethylamine and *N*-benzyl β-D-galactopyranosylcarboxamide, like *N*-benzoyl β-D-galactopyranosylmethylamine, could be nitrosated under the conditions of White [25] (as shown by the characteristic peaks in the visible spectrum at 410 and 427 nm) all three nitrosoamides decomposed within the time of addition to neutral aqueous buffer. Half-lives of 4—10 min in phosphate buffer at pH 7.9 and 25.0 °C have been estimated for White's peptide-derived nitrosoamides,[24] which are enzyme-activated irreversible inhibitors of chymotrypsin; clearly not much anchimeric assistance by sugar hydroxy-groups need be invoked to explain our results.

Acknowledgements

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