106. *N*-(4-Nitrophenyl)oxamic Acid and Related *N*-Acylanilines Are Non-competitive Inhibitors of *Vibrio cholerae* Sialidase but Do Not Inhibit *Trypanosoma cruzi* or *Trypanosoma brucei* Trans-Sialidases

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N-(4-Nitrophenyl)oxamic acid **1**, *N*-(2-fluoro-4-nitrophenyl)oxamic acid **7**, *N*-(4-nitrophenyl)-trifluoroacetamide **3**, and *N*-(2-methoxy-4-nitrophenyl)trifluoroacetamide **9** are non-competitive inhibitors of *Vibrio cholerae* sialidase with K_i-values ranging from 2.66 to 5.18 · 10^{-4} M. These compounds, and the *N*-acetylneuraminic-acid analogues **11**–**13** do not inhibit the sialidase and trans-sialidase activities from *Trypanosoma cruzi*; nor does *N*-(4-nitrophenyl)oxamic acid (**1**) inhibit the corresponding enzyme activities from *T. brucei*.

In 1966, *Edmond et al.* [1] described a number of *N*-aryl- and *N*-heteroaryloxamic acids as inhibitors of influenza virus (strain A2/Eng./1/61) sialidase, and *N*-(4-nitrophenyl)oxamic acid (**1**) was reported to be a competitive inhibitor of *Vibrio cholerae* sialidase [2]. Similar to the competitive inhibitor *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid (DANA; **10**) which inhibits sialidases of various origin [3], **1** might be a lead compound for finding structurally simple sialidase inhibitors with antiviral or anti-trypanosomal properties.

It is surprising that **1** should be a competitive inhibitor of sialidases, given the structural differences between **1** and the transition-state analogue inhibitor **10**. Even if one considers the NO_2 group as an equivalent of the COOH group and the benzene ring as an equivalent of the pyran ring of **10**, or of *N*-acetylneuraminic acid (Neu5Ac), the distance between the nitro- and the *N*-acyl group being more or less equivalent to the distance between the COOH group and the 5-NHAc group in **10** or in Neu5Ac [1], there is no equivalent of the glycerol side chain. A shortening [4–6], deletion [7], or configurational

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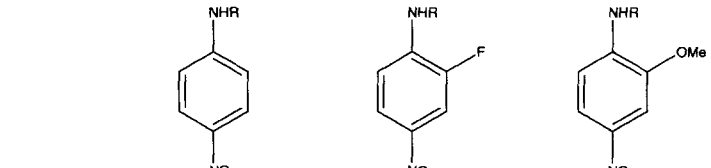
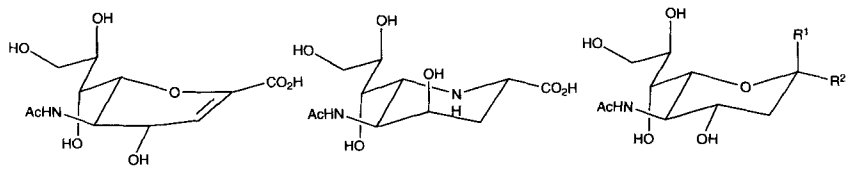


1 R = $COCO_2H$
2 R = $COCO_2Et$
3 R = $COCF_3$
4 R = $COCH_2I$

5 R = H
6 R = $COCO_2Et$
7 R = $COCO_2H$

8 R = H
9 R = $COCF_3$



10

11

12 $R^1 = PO_3H_2$, R^2 = H
13 R^1 = H, $R^2 = PO_3H_2$

change resulting in a change of the conformation [8] of the side chain of Neu5Ac derivatives and analogues is known to strongly weaken binding and inhibiton of *V. cholerae* and other sialidases. *N*-Aryloxamic acids have, however, been used to immobilize sialidases from *Clostridium perfringens* [9] [3], *V. cholerae* [9–11], *Trichomonas fetus*, [11] [12], *Streptococcus pneumoniae* [11], porcine kidney [11], and of viral origin [9] [13] (see [3] for a review). *Brossmer et al.* [11] reported that several sialidases immobilized on aminohexylsepharose 4B carrying *N*-(4-nitrophenyl)oxamoyl substituents are still active, and postulated a hydrophobic interaction between sialidases and immobilized **1**. *Miller et al.* have shown that *Arthrobacter sialophilus* sialidase is inhibited by **10**, but not by **1** [14], and, given the competitive nature of the inhibition [2], have concluded that the homogeneous *A. sialophilus* enzyme is considerably different from the *V. cholerae* enzyme.

Clearly, the type of inhibition by *N*-aryloxamic acids is relevant to a comparison of different sialidases; for this reason, and in the context of our interest in sialidase inhibitors [4] [7] [15–21], we have prepared **1** [22] and its analogues **2** [23] [24], **3** [25], **4** [26] [27], **6** (from **5**), **7**, and **9** (from **8**), and examined their mode of inhibition of *V. cholerae* sialidase. We have also examined the influence of **1–4**, **6**, **7**, and **9** on the sialidase and trans-sialidase activity of *Trypanosoma cruzi*, and the influence of **1** on the corresponding enzymes of *T. brucei*. *T. cruzi* is the etiological agent of *Chagas* disease [28] and its trans-sialidase plays a role in cell invasion [29–32], whereas the corresponding enzyme from *T. brucei*, a parasite causing African sleeping sickness [33], protects the cells towards digestive enzymes within the tsetse fly [34–36].

Poor water solubility characterizes all *N*-acyl-4-nitroanilines **1–4**, **6**, **7**, and **9**. As seen from *Dixon* plots for the acids **1** and **7**, and for the 4-nitro-*N*-(trifluoroacetyl)anilines **3** and **9** (*Figs. 1–4*), these compounds are noncompetitive inhibitors of *V. cholerae* sialidase and

interact at a single site (linear inhibition). The ethyl ester **2** showed only slight inhibition up to a concentration of $5.85 \cdot 10^{-6}$ M (data not shown). The inhibition of the iodoacetamide **4** could not be determined, as its water solubility was below $2 \cdot 10^{-5}$ M. The data of **10** for *V. cholerae* sialidase were determined under the same assay conditions and confirmed the competitive nature of the inhibition (*Fig. 5, Table*), whereas the presence of **10** had practically no effect on the activity of both trypanosomal sialidases (data not shown).

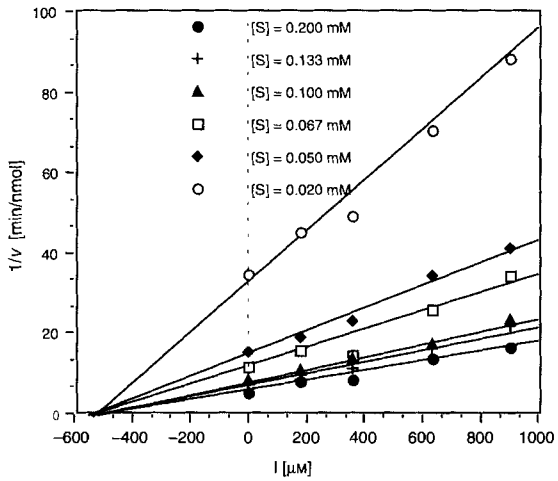


Fig. 1. Dixon *plot of* **1**

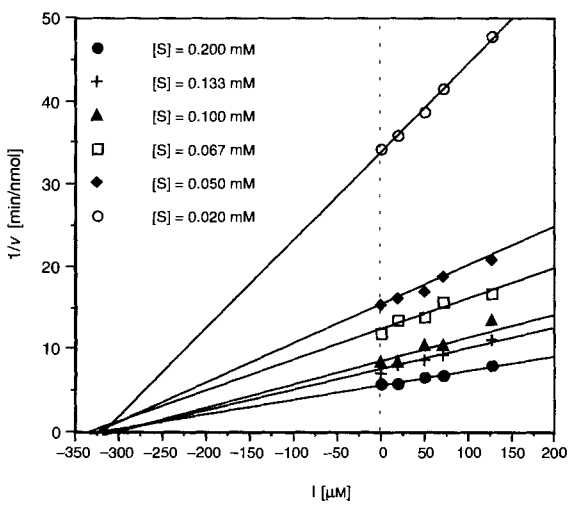


Fig. 2. Dixon *plot of* **3**

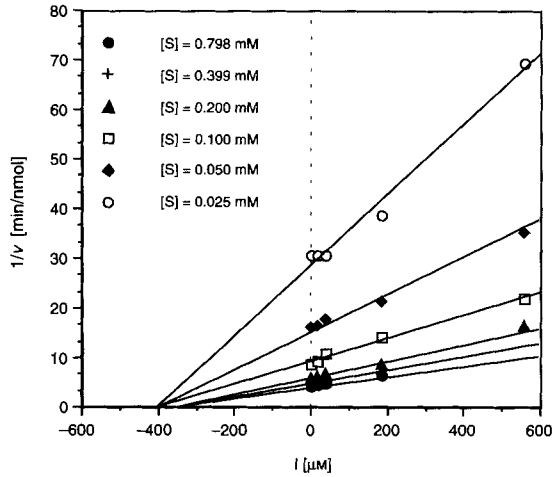


Fig. 3. Dixon *plot of* **7**

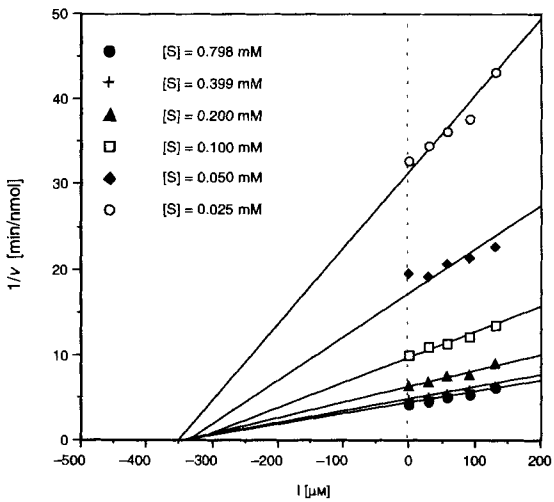


Fig. 4. Dixon *plot of* **9**

Table. *Inhibition of the Sialidase of V.* cholerae

Compound	Type of inhibition	$K_i \cdot 10^{-4}$ [M]
1	non-competitive	3.40 ± 0.12
2	—	—
3	non-competitive	5.18 ± 0.78
4	—	—
7	non-competitive	3.05 ± 0.07
9	non-competitive	2.66 ± 0.02
10	competitive	0.226 ± 0.002

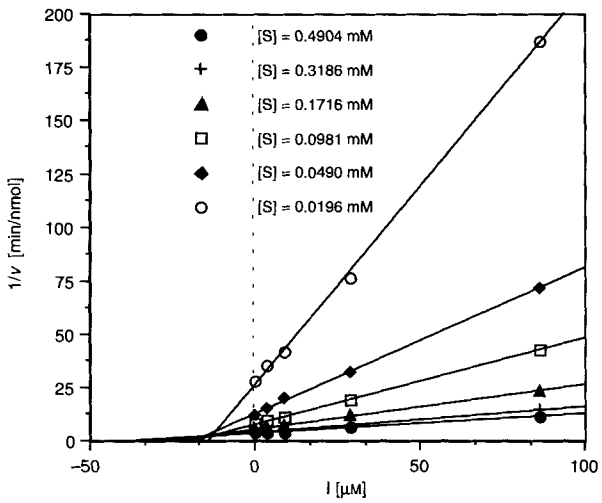


Fig. 5. Dixon *plot of* **10**

In keeping with the finding, that compounds **1**, **3**, **7**, and **9** inhibit the *V. cholerae* enzyme in a non-competitive mode, *N*-acylanilines **1–3** did not inhibit the trans-sialidase of *T. cruzi*, nor did **1** inhibit the corresponding enzyme of *T. brucei*. For reasons of comparison, we also studied the inhibition of the *T. cruzi* enzyme by the Neu5Ac analogues **11** [20], **12**, and **13** [17], competitive inhibitors of the *V. cholerae*, *A. sialophilus*, and *Influenza* sialidase [37], and found no inhibition either (data not shown). Although it is surprising that *N*-acylanilines **3** and **9**, which lack a COOH functionality, are equally good inhibitors as the oxamic acids **1** and **7**, this result is in agreement with the non-competitive mode of inhibition by these compounds where hydrophobic interactions may be important. The elucidation of the exact binding site might be useful for promoting the construction of a novel class of high-affinity *V. cholerae* sialidase inhibitors.

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Experimental Part

Ethyl N-(2-Fluoro-4-nitrophenyl)oxamate (**6**). A soln. of Et_3N (2.0 ml, 1.452 g, 14.34 mmol), ethyl oxalyl chloride (= ethyl 2-chloro-2-oxoacetate; 1.5 ml, 1.84 g, 13.48 mmol) and 2-fluoro-4-nitro-aniline (**5**; 1.086 g, 9.69 mmol) in CH_2Cl_2 (50 ml) was stirred for 12 h at r.t., extracted with 2 × 50 ml of 5% aq. HCl and 50 ml of brine, and dried ($MgSO_4$). FC (80 g of silica gel, Et_2O/hexane 1:1) of the residue obtained after evaporation yielded yellow **6** (1.694 g, 95%). M.p. 154.5–156°. R_f (hexane/Et_2O 1:1) 0.29. IR (KBr): 3377*s*, 3132*w*, 3099*m*, 3086*m*, 3066*m*, 2999*w*, 2949*w*, 2853*w*, 2623*w*, 1943*w*, 1723*s*, 1619*s*, 1561*s*, 1514*s*, 1488*m*, 1452*m*, 1428*m*, 1374*m*, 1351*s*, 1310*s*, 1284*m*, 1245*m*, 1195*m*, 1174*s*, 1160*m*, 1125*m*, 1073*m*, 1020*m*, 948*w*, 930*m*, 899*m*, 871*w*, 848*m*, 812*s*, 770*w*, 745*m*, 712*w*, 658*m*, 548*w*, 521*m*, 455*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 9.35 (*s*, HN); 8.67 (*dd*, *J* = 7.7, 9.0, H–C(6)); 8.14 (*ddd*, *J* = 7.8, 9.0, 10.6, H–C(5)); 8.07 (*dd*, *J* = 2.5, 10.5, H–C(3)); 4.47 (*q*, *J* = 7.2, CH_2); 1.46 (*t*, *J* = 7.2, Me). ^{13}C-NMR (50 MHz, $CDCl_3$): 159.7 (COO); 154.2 (CON); 148.7 (s, C(4)); 130.9 (*s*,

C(1)); 120.8 (*d*, C(6)); 120.5 (*d*, C(5)); 111.5 (C(2)); 111.0 (*d*, C(3)); 64.3 (*t*, CH_2); 13.9 (*q*, Me). EI-MS: 30 (17), 44 (24), 82 (11), 126 (13), 149 (13), 156 (33), 169 (15), 182 (13), 183 (95), 184 (30), 256 (100, M^+). Anal. calc. for $C_{10}H_9FN_2O_5$ (256.19): C 46.88, H 3.54, N 10.93; found: C 47.02, H 3.70, N 11.10.

N-(2-Fluoro-4-nitrophenyl)oxamic Acid (**7**). A soln. of **6** (1.968 g, 7.68 mmol) in THF (80 ml) was treated with a soln. of KOH (1.116 g, 19.91 mmol) in H_2O (162 mg, 9 mmol) and in EtOH (18 mmol). The orange precipitate was removed by filtration, dissolved in H_2O, and treated with 10% aq. HCl until pH 2. Extraction with AcOEt (3 × 700 ml), washing with H_2O, drying, evaporation, and recrystallization of the spontaneously crystallizing residue in MeOH gave **7** (1.084 g, 62%). M.p. 152–154.5°. IR (KBr): 3585*w*, 3337*s*, 3116*m*, 3091*m*, 2924*m*, 2623*w*, 2529*w*, 1942*w*, 1772*s*, 1716*s*, 1698*s*, 1621*s*, 1608*m*, 1557*s*, 1521*s*, 1490*m*, 1432*m*, 1352*s*, 1326*s*, 1289*s*, 1248*m*, 1202*m*, 1173*m*, 1128*m*, 1074*m*, 946*m*, 926*m*, 902*m*, 888*m*, 848*w*, 808*s*, 768*w*, 743*s*, 677*m*, 656*m*, 647*m*, 563*w*, 524*w*, 490*w*, 461*w*. ^{1}H-NMR (300 MHz, (D_6)DMSO): 10.73 (s, NH); 8.25 (*dd*, *J* = 2.5, 10.5, H–C(6)); 8.16 (*dd*, *J* = 2.4, 9.1, H–C(5)); 8.08 (*m*, H–C(3)). ^{13}C-NMR (50 MHz, (D_6)DMSO): 161.2 (COO); 157.5 (CON); 155.5 (*s*, C(2)); 150.5 (*s*, C(1)); 144.4 (*s*, C(4)); 124.7 (*d*, C(6)); 120.3 (*d*, C(5)); 111.8 (*d*, C(3)). EI-MS: 57 (22), 63 (23), 82 (28), 83 (33), 90 (21), 183 (100), 228 (63, M^+). Anal. calc. for $C_8H_5FN_2O_5$ (228.14): C 42.12, H 2.21, N 12.28; found: C 42.31, H 2.40, N 12.10.

2,2,2-Trifluoro-N-(2-methoxy-4-nitrophenyl)acetamide (**9**) was prepared by analogy to a procedure in [25]. A mixture of 2-methoxy-4-nitroaniline (**8**, 320 mg, 1.9 mmol), Et_3N (0.27 ml, 196 mg, 1.94 mmol), $(CF_3CO)_2O$ (0.27 ml, 407 mg, 1.94 mmol), and CH_2Cl_2 (25 ml) was stirred for 12 h at 8–16° under N_2. Extraction with 5% HCl (25 ml) and brine (25 ml), drying of the org. phase, and evaporation, followed by recrystallization of the residue in CH_2Cl_2/hexane gave slightly yellow **9** (437.2 mg, 88%). M.p. 110.5–111°. IR (KBr): 3362*s*, 3100*m*, 2997*w*, 2960*w*, 2856*w*, 1739*s*, 1624*m*, 1592*s*, 1553*s*, 1518*s*, 1494*s*, 1470*m*, 1451*m*, 1421*m*, 1347*s*, 1309*m*, 1278*s*, 1260*s*, 1227*s*, 1202*s*, 1172*s*, 1146*s*, 1123*s*, 1096*m*, 1028*s*, 900*m*, 879*m*, 835*m*, 824*w*, 800*m*, 761*w*, 746*m*, 734*m*, 654*m*, 596*w*, 517*w*, 460*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.70 (*s*, NH); 8.53 (*d*, *J* = 9.0, H–C(6)); 7.97 (*dd*, *J* = 2.3, 9.0, H–C(5)); 7.82 (*d*, *J* = 2.3, H–C(3)); 4.07 (*s*, MeO). ^{13}C-NMR: 154.8 (CF_3); 148.2 (C=O); 144.8 (*s*, C(2)); 130.7 (*s*, C(1)); 123.9 (*s*, C(4)); 119.3 (*d*, C(6)); 117.2 (*d*, C(5)); 105.5 (*d*, C(3)); 56.7 (*q*, MeO). EI-MS: 69 (12), 78 (11), 195 (34, $[M - CF_3]^+$), 203 (14), 264 (100, M^+), 265 (10, $[M+1]^+$) . Anal. calc. for $C_9H_7F_3N_2O_4$ (264.16): C 40.92, H 2.67, N 10.60; found: C 41.10, H 2.65, N 10.37.

Assays. A) *Trypanosoma cruzi.* 1. *Trans-Sialidase Activity.* The enzyme was purified from the supernatant of a culture of *T. cruzi* (Tulahuen 2 strain)-infected Vero cells [38] by affinity chromatography through a *Sepharose 4-B* column (*Pharmacia*) containing immobilized monospecific antibodies [39]. The buffer (1M Tris (= tris-(hydroxymethyl)aminomethane = 2-amino-2-(hydroxymethyl)propane-1,3-diol), pH 7.4) in which the enzyme was obtained was changed to 30 mM Pipes (= piperazine-1,4-bis(ethanesulfonic acid)) pH 7.0 by passage through a *Sephadex G-25* column (*Pharmacia*). The amount of enzyme used per assay was that obtained from *ca.* 10^4 parasites. The incubation mixtures contained, in a total volume of 50 µl, 1 µM [*galactose*-^{14}C]-*N*-acetyllactosamine (285 Ci/mol) [40], 50 µM sialyllactose (*Sigma*), 50 mM Pipes (pH 7.0), 1 mM inhibitor dissolved in DMF, and 10 µl of the enzyme soln. above. Control experiments contained DMF devoid of inhibitor, blanks were run without enzyme. Incubations were kept at 37° for 30 min, and then treated with H_2O (1 ml) and *QAE-Sephadex* slurry (0.1 ml; *Pharmacia*). The beads were washed with H_2O (1 ml) and treated with 1M $NH_4(HCOO)$ (1 ml). After adding *Bray's* solution (3 ml) [41] to the $NH_4(HCOO)$ eluate, radioactivity was determined in a liquid scintillation counter. Trans-sialidase activity is expressed in dpm of the supernatant minus the blank value of 187 dpm.

2. *Sialidase Activity.* The incubation mixtures contained, in a total volume of 20 µl, enzyme as above (10 µl), 80 µM (4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (= *N*-acetyl-2-*O*-(4-methylumbelliferyl)-α-D-neuraminic acid; Mu-Neu5Ac; *Sigma*), and 1 mM of the inhibitor in a final Pipes buffer (pH 7.0) concentration of 15 mM. Controls had DMF containing no inhibitor, blanks were obtained by adding 10 µl of 30 mM Pipes buffer (pH 7.0) instead of the enzyme. After 70 min of incubation time, reactions were stopped by addition of 3 ml of 100 mM Tris buffer (pH 11.3) and fluorescence of the liberated 4-methylumbelliferone was measured on a *JASCO* fluorescence spectrophotometer (excitation at 366 and emission at 448 nm, *cf.* [40] [42]).

B) *Trypanosoma brucei.* Freshly prepared lysates of procyclic culture forms of *T. brucei* EATRO 427 were used as enzyme source. 1. *Trans-sialidase Activity.* Enriched enzyme (1 mU; 0.57 U sialidase/mg protein) was incubated in a total volume of 100 µl, containing 3.7 nmol of [D-*glucose*-1-^{14}C-]lactose (54.3 mCi/mmol; *Amersham Buchler*, Braunschweig, Germany), 1 mM Neu5Ac-α(2,3)-lactose, 4 mM lactose, 50 mM Bis-Tris

buffer (pH 6.8), and 0–10 mM *N*-(4-nitrophenyl)oxamic acid (**1**) dissolved in 70% EtOH (the slight inhibition by EtOH was accounted for in the results). After 60 min at 37°, H_2O (1 ml) was added, followed by passage through Q-Sepharose fast-flow (1 ml; *Pharmacia*) equilibrated and run in H_2O. Trans-sialidase activity is expressed in dpm eluted from the column with 1 ml of 1M $NH_4(HCOO)$ [43] minus the blank value of 70 dpm. In the absence of the enzyme, a 20 mM soln. of **1** had no influence on the result.

2. *Sialidase activity* [44]. Incubations were essentially as described above for the trans-sialidase, but no radioactive lactose was added. The amount of sialic acid released by the action of *T. brucei* sialidase was measured using the microadaption of the periodic acid/thiobarbituric acid assay [45] and is expressed in nmol of free sialic acid. Blanks were run in the presence of heat-denatured enzyme.

C) *Vibrio cholerae*. The kinetic inhibition parameters were determined with a spectrofluorimetric assay, using (4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Mu-Neu5Ac) [46], as described by *Warner* and *O'Brien* [47] and *Potier et al.* [48], and following mainly the variant of *Schauer et al.* [8]. The sialidase was from *Boehringer Mannheim* (Mannheim, Germany) in protease-free quality, Mu-Neu5Ac was from *Fluka* (Buchs, Switzerland). All other chemicals were of biochemical grade unless stated otherwise. Prior to use, 0.1 U of the enzyme was dissolved in 10 ml of 0.1M acetate buffer of pH 5.5 (0.5 mM $CaCl_2$, 0.01% (*w/v*) NaN_3), containing 0.1 mg/ml bovine serum albumin (*Boehringer Mannheim*). Incubations were carried out at 37° in a total volume of 100 µl containing 0.2 mU enzyme (20 µl of the above soln.), a final $CaCl_2$ concentration of 0.5mM, 0.1M acetate buffer (pH 5.5), an aq. soln. of Mu-Neu5Ac (6 different concentrations, ranging typically from 2 K_M to 0.1 K_M), and an aq. soln. of the inhibitor (4 different concentrations). Controls did not contain inhibitor, blanks were run without enzyme. As a rule, inhibitors were preincubated with the enzyme for 10 min before starting the enzymatic reaction by the addition of substrate. After 15 min, the reaction was stopped by rapid addition of 900 µl of cold glycine buffer of pH 10 (0.06M NaCl, 0.042M Na_2CO_3, 0.133M glycine) and liberated 4-methylumbelliferone (Mu) immediately determined on a *Perkin-Elmer MPF-66* fluorescence spectrophotometer using a wavelength of 365 ± 0.6 nm for excitation and of 450 ± 0.6 nm for emission[2]) (integration time 2 s). Fluorescence readings were converted into concentration values by means of a standard curve[3]). The Mu used for establishing this curve was purified according to [53]; its purity was checked by microanalysis. Blank values

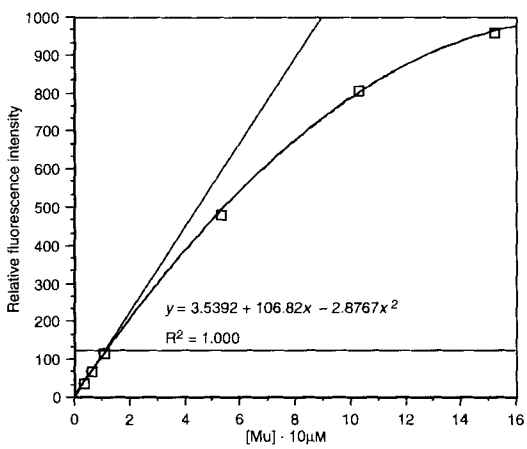

Fig. 6. *4-Methylumbelliferone* (Mu) *standard curve*

[2]) It had been shown [49] that plots of liberated Neu5Ac *vs.* time are essentially linear over the 15-min assay period.

[3]) As illustrated by *Fig. 6*, a linear dependence of fluorescence intensity is realized only for a very narrow range of Mu concentrations. The simplification of substituting 1/velocity by 1/fluorescence as applied recently [50–52] may, therefore, constitute a source of considerable error.

to account for nonenzymatic hydrolysis were subtracted from the values obtained in the presence of enzyme before calculating the amount of nmoles of Neu5Ac released. *Systematically* weighted linear regression analysis of the double reciprocal form of the *Michaelis-Menten* relation provided provisional estimates of the kinetic parameters ($K_M°$, $v_{max}°$). Exact values with standard errors were obtained by multiple *Taylor* expansion of the hyperbolic form of the *Michaelis-Menten* equation around $x = K_M°$ [54]. Iteration cycles were repeated until consecutive results differed in less than 10^{-3}%. In a secondary plot, the obtained K_M values (for *competitive* inhibition) resp. $1/v_{max}$ values (for *noncompetitive* inhibition) were plotted against the inhibitor concentration. *Individually* weighted ($p_i \sim 1/\sigma_i^2$) linear regression analysis then yielded the corresponding K_i values (extrapolated intercept with the abscissa) together with confidence intervals containing error correlation from both linear regression parameters. The K_i value for **10**, determined under the same conditions, was $2.26 \pm 0.02 \cdot 10^{-5}$ M ($1.6 \cdot 10^{-5}$ M [16] [55], $2.5 \cdot 10^{-5}$ M [56]), and the average K_M (*weighted mean* of all experiments) $2.11 \pm 0.03 \cdot 10^{-4}$ M ($2.0 \cdot 10^{-4}$ M [20]). No significant fluorescence quenching was observed for the inhibitor concentrations employed in the assay. Results are summarized in the *Table*.

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