APPENDIX C

Illkirch, May 24, 2005

FLUORESCENCE INVESTIGATION OF THE INTERACTION BETWEEN HUMAN ANTITHROMBIN III AND 13 NEW OLIGOSACCHARIDE DERIVATIVES

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INTRODUCTION

Antithrombin III (AT III), a protein that belongs to the serpin superfamily of the serine proteinase inhibitors, plays a critical role in the blood coagulation. AT III and the other inhibitory serpins share the property of forming irreversible complexes with their target enzymes through a unique suicide substrate-like inhibition mechanism that leads to a covalent acyl-enzyme complex (Gettins 2002). AT III is the main inhibitor of thrombin and factor Xa, two enzymes involved in the coagulation cascade and that react at low rate with this serpin (Craig et al. 1989). The highly negatively charged polysaccharide heparin is known to dramatically enhance the rate of inhibition of factor Xa and thrombin by AT III through its binding to the serpin *via* a specific pentasaccharide fragment present in about one third of the naturally occurring heparin chains (Choay et al. 1983; Olson et al. 1992), a property widely used for the therapeutic modulation of blood coagulation. The pentasaccharide-promoted acceleration of factor Xa inhibition process results from a conformational change of AT III that extrudes its reactive site loop from a partially buried position to a more accessible one (Huntington et al. 1996; Jin et al. 1997; Olson et al. 1992) whereas a bridging mechanism that requires longer heparin chains mainly accounts for the acceleration of thrombin inhibition (Olson and Björk 1991).

The heparin/pentasaccharide-induced structure modification of AT III has been investigated by numerous spectroscopic techniques. It has been published that the AT III intrinsic fluorescence increases by 30-40 % when it binds to high affinity heparin or to the specific pentasaccharide. This property has been used by many investigators to quantitate the affinity of these compounds for AT III as well as to investigate by fast kinetic studies the mechanism by which they associate with the serpin (Desai et al. 1998; Huntington et al. 1996; Olson and Shore 1981; Olson et al. 1981; Schedin-Weiss et al. 2002).

We report here the preliminary single blind investigation of the AT III-binding properties of 13 unknown enoxaparin-derived low molecular weight oligosaccharides with therapeutic potential by monitoring the alteration of the tryptophan fluorescence of the protein upon its reaction with these products.

MATERIAL

13 oligosaccharides numbered from 1 to 13 and the heparin pentasaccharide were provided by Dr. C. Viskov (Sanofi-Aventis, Vitry-sur-Seine, France). The molecular mass of each product was known before the analyses. The pure human AT III preparation is a product from Hyphen BioMed (Neuville-sur-Oise, France). Its concentration in the stock solution was calculated from its absorbance at 280 nm using $\varepsilon_{280nm} = 35560\ M^{-1}.cm^{-1}$. The serpin solution was found to contain at least 95 % of active protein as judged by its activity against active site-titrated porcine trypsin (Boudier and Bieth 2001). All other reagents are of analytical grade.

EXPERIMENTAL PROCEDURES

The affinity and binding stoichiometry governing the interaction of the unknown oligosaccharide derivatives for antithrombin (AT III) have been assessed at 25° C in 0.05 M hepes containing 0.1 M NaCl by equilibrium titration experiments in which the fluorescence of the tryptophan residues of the serpin was monitored when reacting constant concentrations of the protein with increasing concentrations of these compounds, a procedure already described by others (Desai et al. 1998; Huntington et al. 1996; Lin et al. 2001; Olson and Shore 1981). As a reference product we used the heparin pentasaccharide.

The fluorescence intensities measurements (λ_{ex} = 280 nm, 5 nm band-pass, λ_{em} = 340 nm, 5 nm band-pass) were performed using a RF5000 Shimadzu fluorospectrophotometer equipped with a thermostated cell holder. A typical fluorescence equilibrium titration experiment was done as follows: an aliquot of the buffered AT III stock solution was diluted (final concentration = 0.183, 0.091 or 0.078 μM) into the buffer contained in a 1 cm path fluorometer cuvette (final volume = 2.5 ml) and the fluorescence intensity was read at 340 nm prior consecutive additions of small volumes (0.5 – 2.0 μl) of the appropriate oligosaccharide solution. Before addition of AT III, the apparatus was set to zero by reading against the buffer alone.

The stock and working solutions of the ligands were prepared by dilution of their powder in the hepes buffer as indicated (see table I in annex). Their concentrations were

calculated from their absorbance at 232 nm and $\varepsilon_{232nm} = 5400\ M^{-1}.cm^{-1}$ except for the heparin pentasaccharide which concentration was obtained by weight and Mr = 1818 Da.

The best values of K_d, the equilibrium dissociation constant of the oligosaccharide : AT III complexes and n, the binding stoichiometry (ie the ratio of the concentration of the high affinity species present in the reaction mixture *vs* the total concentration of the studied product) were obtained by non-linear least square fitting of the fluorescence data to the following classical binding equation:

$$\Delta f = \Delta f_{max} \frac{\left([ATIII]_0 + n[ols]_0 + K_d\right) - \sqrt{\left([ATIII]_0 + n[ols]_0 + K_d\right)^2 - 4n[ols]_0[ATIII]_0}}{2[ATIII]_0} \quad (1)$$

where $[ATIII]_0$ and $[ols]_0$ stand for the AT III and oligosaccharide initial concentrations, respectively, and Δf and Δf_{max} are the absolute change of tryptophan fluorescence intensity for a given ligand concentration and that of the maximum fluorescence change, respectively. Non-linear regression analysis was done with the GraFit software (Erithacus Software).

RESULTS

The reaction of saturating concentrations of the oligosaccharide products with AT III was accompanied by a 24.8 – 47.0 % increase of the intrinsic fluorescence of the protein. We made use of this fluorescence-enhancing effect to generate saturation curves by reacting incremental amounts of oligosaccharide with AT III. The fluorescence data sets were analyzed as described above to assess K_d and n. The figures (provided in the annex) show that the theoretical curves generated using equation 1 and the best estimates of these parameters adequately fit the experimental data. The values of K_d, n and the maximal fluorescence increase for each AT III : oligosaccharide pair are summarized in table I. Their standard error and the concentration of AT III used in each experiment are given with the corresponding figure (see annex).

TABLE I

Equilibrium dissociation constants (K_d), binding stoichiometries (n) and maximal fluorescence increases (ΔF_{max}) for the AT III : oligosaccharide interactions

product	K_d (nM)	n (M/M)[a]	ΔF_{max} (%)[b]
1	1.2	0.18	24.8
2	33.9	0.91	47.0
3	5.4	0.93	32.4
4	14.8	0.92	40.3
5	3.6	0.48	34.4
6	47.1	1.1	32.7
7	42.8	1.22	39.9
8	12.9	1.10	33.8
9	9.4	1.04	38.4
10	11.0	0.83	34.5
11	17.7	0.96	29.7
12	10.4	0.11	38.0
13	3.6	0.11	32.2
pentasaccharide	25.4	1.02	35.7

[a]n = binding stoichiometry: ratio of the concentration of the high the affinity species present in the reaction mixture vs the total concentration of the studied product. The calculation here assumes that for a given compound, all the AT III-binding species interact with the same affinity with the serpin.
[b]calculated from the fluorescence intensities measured in the presence of saturating oligosaccharide concentrations.

CONCLUSION

We investigated here the AT III-binding properties of 13 unknown oligosaccharides at 25° C in 0.05 M hepes buffer containing 0.1 M NaCl. For the purpose of comparison, the heparin binding pentasaccharide was submitted to the same analyses. The interaction of AT III with the various products was fluorometrically followed by monitoring at 340 nm the intrinsic fluorescence of AT III with excitation at 280 nm. The reactions were accompanied by a fluorescence increase ranging from 24.8 % (product 1) to 47.0 % (product 2). The

heparin pentasaccharide induced a 35.7 % enhancement. Similar values have been found for high affinity heparin (Olson and Shore 1981) and small oligosaccharides (Desai et al. 1998).

The 13 unknown derivatives reveal to strongly bind AT III as indicated by the K_d values listed above in table I. Under the experimental conditions used, 10 products exhibit a tighter AT III-binding behaviour than that found for the pentasaccharide (K_d = 25.4 nM). Similar affinities have been reported for the interaction of synthetic oligosaccharides with AT III (Petitou et al. 1999a; Petitou et al. 1999b). The equilibrium dissociation constants for the complexes of products 2, 6 and 7 with the serpin (K_d = 33.9, 47.1 and 42.8 nM, respectively) compare with K_d for the pentasaccharide. From the affinity point of view product 1 is the best (K_d = 1.2 nM).

Products 1-11, and the heparin-binding pentasaccharide, are chemically well defined derivatives with precisely known molecular mass. For most of these compounds (except products 1 and 5), the experimentally determined binding stoichiometry for their complex with AT III is ranging from 0.83 to1.22 in agreement with the hypothesis that the oligosaccharide material contained in their preparations is composed of a single high affinity species only. The reason why products 1 and 5 interact with AT III according to a reaction stoichiometry significantly different from 1 remains unclear.

Products 12 and 13 are non homogeneous oligosaccharides with an average molecular weight of 4300 and 4100 Da, respectively. Fluorescence titration data analysis indicates that the AT III-binding molecules contained in both preparations form tight complexes with AT III (K_d = 10.4 and 3.6 nM, respectively) but account for 11% (M/M) of the total oligosaccharide material only, a fraction of high affinity components close to that reported for enoxaparin (12.5 %, Lin et al. 2001).

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- ANNEX -

to

FLUORESCENCE INVESTIGATION OF THE INTERACTION BETWEEN HUMAN ANTITHROMBIN III AND 13 NEW OLIGOSACCHARIDE DERIVATIVES

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TABLE I

Quantities available, molecular mass and concentrations in their solutions of the oligosaccharide compounds used in this study

product	Mr	quantity of powder available (mg)	diluted in: (µl) [a]	product concentration in the stok solution (mM) [b]	product concentration in the working solution (mM) [c]
1	3165	0.1	100	0.161	0.161
2	3165	3	1000	0.182	0.182
3	3165	1.3	1000	0.097	0.032
4	3165	1.1	500	0.133	0.133
5	3045	1.9	500	0.084	0.042
6	3165	2.7	500	0.114	0.114
7	3063	1.9	500	0.213	0.213
8	3003	2	500	0.203	0.203
9	2883	1	350	0.100	0.100
10	2337.5	2.3	500	2.030	0.0845
11	1834	1.5	500	1.350	0.135
12	4300	17 (2.43 used)	500	1.270	1.270
13	4100	17 (2.47 used)	500	1.440	1.440
pentasaccharide	1818	4.6	500	5.060	0.101

[a] quantity of hepes buffer added to the vial to make the stock solution.
[b] detemined from $A_{232\ nm}$ and $\varepsilon = 5400\ M^{-1}.cm^{-1}$ except for the pentasaccharide, the concentration of which was calculated from the weight and the Mr.
[c] solutions used to perform the equilibrium titration experiments.

PRODUCT 1

	1	2	3	4
Type				
Column Name	microliter of working solution	[product 1] (micro M)	F (arbit units)	delta F (arbit units)
1	0.0000	0.0000	298.0000	0.0000
2	0.5000	0.0322	300.0000	2.0000
3	1.0000	0.0644	307.0000	9.0000
4	2.0000	0.1288	317.0000	19.0000
5	3.0000	0.1932	325.0000	27.0000
6	4.0000	0.2576	336.0000	38.0000
7	5.0000	0.3220	348.0000	50.0000
8	6.0000	0.3864	352.0000	54.0000
9	7.0000	0.4508	360.0000	62.0000
10	8.0000	0.5152	365.0000	67.0000
11	9.0000	0.5796	368.0000	70.0000
12	10.0000	0.6440	369.0000	71.0000
13	12.0000	0.7728	371.0000	73.0000
14	14.0000	0.9016	371.0000	73.0000
15	16.0000	1.0304	371.0000	73.0000
16	18.0000	1.1592	372.0000	74.0000
17	20.0000	1.2880	372.0000	74.0000
18	22.0000	1.4168	372.0000	74.0000
19	24.0000	1.5456	372.0000	74.0000
20	26.0000	1.6744	372.0000	74.0000

PRODUCT 2

	1	2	3	4
Type				
Column Name	microliter of working solution	fluo (arbitr units)	delta F (arbitr units)	[product 2] (microM)
1	0.0000	342.0000	0.0000	0.0000
2	1.0000	394.0000	52.0000	0.0735
3	2.0000	432.0000	90.0000	0.1471
4	3.0000	449.0000	107.0000	0.2206
5	4.0000	465.0000	123.0000	0.2942
6	5.0000	478.0000	136.0000	0.3677
7	6.0000	487.0000	145.0000	0.4413
8	7.0000	492.0000	150.0000	0.5148
9	8.0000	493.0000	151.0000	0.5884
10	9.0000	496.0000	154.0000	0.6619
11	10.0000	498.0000	156.0000	0.7354
12	11.0000	500.0000	158.0000	0.8090
13	13.0000	500.0000	158.0000	0.9561
14	15.0000	502.0000	160.0000	1.1032
15	17.0000	503.0000	161.0000	1.2502
16	19.0000	502.0000	160.0000	1.3973
17	21.0000	503.0000	161.0000	1.5444

PRODUCT 3

	1	2	3	4
Type				
Column Name	volume (micro l)	[product 3] (micro M)	fluorescence (arbit units)	delta fluo (arbit units)
1	0.0000	0.0000	222.0000	0.0000
2	1.0000	0.0129	230.0000	8.0000
3	2.0000	0.0259	241.0000	19.0000
4	3.0000	0.0388	253.0000	31.0000
5	4.0000	0.0517	264.0000	42.0000
6	5.0000	0.0646	270.0000	48.0000
7	6.0000	0.0776	276.0000	54.0000
8	7.0000	0.0905	280.0000	58.0000
9	8.0000	0.1034	286.0000	64.0000
10	9.0000	0.1164	288.0000	66.0000
11	10.0000	0.1293	289.0000	67.0000
12	11.0000	0.1422	289.0000	67.0000
13	13.0000	0.1681	290.0000	68.0000
14	15.0000	0.1940	292.0000	70.0000
15	17.0000	0.2198	294.0000	72.0000
16	19.0000	0.2457	294.0000	72.0000
17	21.0000	0.2716	294.0000	72.0000
18	23.0000	0.2974	294.0000	72.0000

PRODUCT 4

	1	2	3	4
Type				
Column Name	microliter of working solution	F (arbit units)	delta F (arbit units)	[product 4] (micro M)
1	0.0000	258.0000	0.0000	0.0000
2	1.0000	303.0000	45.0000	0.0532
3	2.0000	332.0000	74.0000	0.1064
4	3.0000	342.0000	84.0000	0.1596
5	4.0000	351.0000	93.0000	0.2128
6	5.0000	354.0000	96.0000	0.2660
7	6.0000	356.0000	98.0000	0.3192
8	7.0000	358.0000	100.0000	0.3724
9	8.0000	359.0000	101.0000	0.4256
10	9.0000	360.0000	102.0000	0.4788
11	10.0000	361.0000	103.0000	0.5320
12	11.0000	361.0000	103.0000	0.5852
13	12.0000	361.0000	103.0000	0.6384
14	14.0000	362.0000	104.0000	0.7448
15	16.0000	361.0000	103.0000	0.8512

PRODUCT 5

	1	2	3	4
Type				
Column Name	Volume (micro l)	fluorescence (arbit units)	[product 5](micro M)	Delta F (arbit units)
1	0.0000	209.0000	0.0000	0.0000
2	1.0000	217.0000	0.0168	8.0000
3	2.0000	223.0000	0.0336	14.0000
4	3.0000	230.0000	0.0504	21.0000
5	4.0000	236.0000	0.0672	27.0000
6	5.0000	246.0000	0.0840	37.0000
7	6.0000	250.0000	0.1008	41.0000
8	7.0000	258.0000	0.1176	49.0000
9	8.0000	263.0000	0.1344	54.0000
10	9.0000	267.0000	0.1512	58.0000
11	10.0000	269.0000	0.1680	60.0000
12	11.0000	271.0000	0.1848	62.0000
13	12.0000	275.0000	0.2016	66.0000
14	13.0000	276.0000	0.2184	67.0000
15	14.0000	277.0000	0.2352	68.0000
16	15.5000	279.0000	0.2604	70.0000
17	17.0000	280.0000	0.2856	71.0000
18	19.0000	280.0000	0.3192	71.0000
19	21.0000	280.0000	0.3528	71.0000
20	23.0000	281.0000	0.3864	72.0000
21	25.0000	281.0000	0.4200	72.0000
22	27.0000	281.0000	0.4536	72.0000
23	29.0000	281.0000	0.4872	72.0000

PRODUCT 6

	1	2	3	4
Type				
Column Name	Volume (micro l)	fluorescence (arbit units)	[product 6] (micro M)	Delta F (arbit units)
1	0.0000	220.0000	0.0000	0.0000
2	1.0000	247.0000	0.0456	27.0000
3	2.0000	260.0000	0.0912	40.0000
4	3.0000	268.0000	0.1368	48.0000
5	4.0000	275.0000	0.1824	55.0000
6	5.0000	276.0000	0.2280	56.0000
7	6.0000	281.0000	0.2736	61.0000
8	7.0000	282.0000	0.3192	62.0000
9	8.0000	285.0000	0.3648	65.0000
10	9.0000	287.0000	0.4104	67.0000
11	10.0000	288.0000	0.4560	68.0000
12	11.0000	288.0000	0.5016	68.0000
13	12.5000	289.0000	0.5700	69.0000
14	14.0000	288.0000	0.6384	68.0000
15	16.0000	290.0000	0.7296	70.0000
16	18.0000	289.0000	0.8208	69.0000
17	20.0000	291.0000	0.9120	71.0000
18	22.0000	289.0000	1.0032	69.0000
19	24.0000	292.0000	1.0944	72.0000
20	26.0000	293.0000	1.1856	73.0000
21	28.0000	292.0000	1.2768	72.0000
22	30.0000	292.0000	1.3680	72.0000

PRODUCT 7

	1	2	3	4
Type				
Column Name	microliter of working solution	F (arbit units)	delta F (arbit units)	[product 7] (micro M)
1	0.0000	258.0000	0.0000	0.0000
2	0.5000	297.0000	39.0000	0.0421
3	1.0000	312.0000	54.0000	0.0842
4	1.5000	328.0000	70.0000	0.1264
5	2.0000	340.0000	82.0000	0.1685
6	2.5000	345.0000	87.0000	0.2106
7	3.0000	347.0000	89.0000	0.2527
8	3.5000	348.0000	90.0000	0.2948
9	4.0000	350.0000	92.0000	0.3370
10	4.5000	352.0000	94.0000	0.3791
11	5.0000	352.0000	94.0000	0.4212
12	6.0000	356.0000	98.0000	0.5054
13	7.0000	356.0000	98.0000	0.5897
14	8.0000	358.0000	100.0000	0.6739
15	9.0000	359.0000	101.0000	0.7582
16	10.0000	360.0000	102.0000	0.8424
17	11.0000	360.0000	102.0000	0.9266
18	12.0000	361.0000	103.0000	1.0109
19	14.0000	361.0000	103.0000	1.1794
20	16.0000	361.0000	103.0000	1.3478

PRODUCT 8

	1	2	3	4
Type				
Column Name	microliter of working solution	F (arbit units)	delta F (arbit units)	[product 8] (micro M)
1	0.0000	245.0000	0.0000	0.0000
2	1.0000	286.0000	41.0000	0.0817
3	2.0000	302.0000	57.0000	0.1633
4	3.0000	321.0000	76.0000	0.2450
5	4.0000	325.0000	80.0000	0.3266
6	5.0000	326.0000	81.0000	0.4083
7	6.0000	327.0000	82.0000	0.4899
8	7.0000	326.0000	81.0000	0.5716
9	9.0000	326.0000	81.0000	0.7349
10	11.0000	328.0000	83.0000	0.8982
11	13.0000	326.0000	81.0000	1.0615
12	15.0000	326.0000	81.0000	1.2248

PRODUCT 9

	1	2	3	4
Type				
Column Name	microliter of working solution	F (arbit units)	delta F (arbit units)	[product 9] (micro M)
1	0.0000	247.0000	0.0000	0.0000
2	1.0000	286.0000	39.0000	0.0399
3	2.0000	310.0000	63.0000	0.0799
4	3.0000	327.0000	80.0000	0.1198
5	4.0000	331.0000	84.0000	0.1597
6	5.0000	335.0000	88.0000	0.1997
7	6.0000	337.0000	90.0000	0.2396
8	7.0000	338.0000	91.0000	0.2796
9	8.0000	340.0000	93.0000	0.3195
10	9.0000	339.0000	92.0000	0.3594
11	10.0000	340.0000	93.0000	0.3994
12	12.0000	340.0000	94.0000	0.4792
13	14.0000	341.0000	95.0000	0.5591
14	16.0000	341.0000	95.0000	0.6390
15	18.0000	340.0000	94.0000	0.7188

PRODUCT 10

	1	2	3	4
Type				
Column Name	Volume (micro l)	fluorescence (arbit units)	[product10] (micro M)	Delta F (arbit units)
1	0.0000	229.0000	0.0000	0.0000
2	1.0000	255.0000	0.0338	26.0000
3	2.0000	273.0000	0.0677	44.0000
4	3.0000	286.0000	0.1015	57.0000
5	4.0000	293.0000	0.1353	64.0000
6	5.0000	302.0000	0.1691	73.0000
7	6.0000	302.0000	0.2030	73.0000
8	7.0000	304.0000	0.2368	75.0000
9	8.0000	305.0000	0.2706	76.0000
10	9.0000	305.0000	0.3045	76.0000
11	10.5000	306.0000	0.3552	77.0000
12	12.0000	307.0000	0.4060	78.0000
13	13.5000	307.0000	0.4567	78.0000
14	15.0000	308.0000	0.5075	79.0000
15	16.5000	308.0000	0.5582	79.0000
16	18.0000	307.0000	0.6090	78.0000
17	19.5000	308.0000	0.6597	79.0000

PRODUCT 11

	1	2	3	4
Type				
Column Name	microliter of working solution	F (arbit units)	delta F (arbit units)	[product 11] (micro M)
1	0.0000	320.0000	0.0000	0.0000
2	0.5000	345.0000	25.0000	0.0271
3	1.0000	360.0000	40.0000	0.0543
4	1.5000	375.0000	55.0000	0.0814
5	2.0000	388.0000	68.0000	0.1086
6	2.5000	391.0000	71.0000	0.1357
7	3.0000	397.0000	77.0000	0.1629
8	3.5000	402.0000	82.0000	0.1900
9	4.0000	403.0000	83.0000	0.2172
10	5.0000	407.0000	87.0000	0.2714
11	6.0000	409.0000	89.0000	0.3257
12	7.0000	412.0000	92.0000	0.3800
13	8.0000	411.0000	91.0000	0.4343
14	9.5000	412.0000	92.0000	0.5157
15	11.0000	413.0000	93.0000	0.5972
16	12.5000	414.0000	94.0000	0.6786
17	14.0000	415.0000	95.0000	0.7600
18	15.5000	414.0000	94.0000	0.8415
19	17.0000	414.0000	94.0000	0.9229
20	18.5000	415.0000	95.0000	1.0043
21	20.0000	414.0000	94.0000	1.0858
22	21.5000	414.0000	94.0000	1.1672

PRODUCT 12

	1	2	3	4
Type				
Column Name	microliter of working solution	[product 12] (micro M)	F (arbit units)	delta F (arbit units)
1	0.0000	0.0000	300.0000	0.0000
2	0.5000	0.2540	335.0000	35.0000
3	1.0000	0.5080	358.0000	58.0000
4	1.5000	0.7620	375.0000	75.0000
5	2.0000	1.0160	389.0000	89.0000
6	2.5000	1.2700	401.0000	101.0000
7	3.0000	1.5240	403.0000	103.0000
8	4.0000	2.0320	407.0000	107.0000
9	5.0000	2.5400	409.0000	109.0000
10	6.0000	3.0480	411.0000	111.0000
11	7.0000	3.5560	412.0000	112.0000
12	8.0000	4.0640	413.0000	113.0000
13	9.0000	4.5720	413.0000	113.0000
14	10.0000	5.0800	413.0000	113.0000
15	11.5000	5.8420	414.0000	114.0000
16	13.0000	6.6040	414.0000	114.0000
17	14.5000	7.3660	414.0000	114.0000
18	16.0000	8.1280	414.0000	114.0000
19	17.5000	8.8900	414.0000	114.0000
20	19.0000	9.6520	414.0000	114.0000
21	20.5000	10.4140	414.0000	114.0000

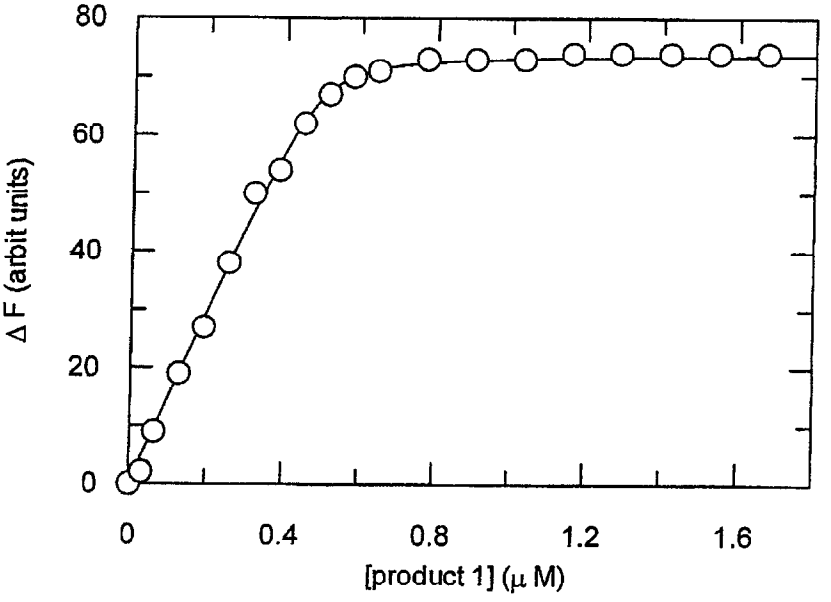
PRODUCT 13

	1	2	3	4
Type				
Column Name	microliter of working solution	[product 13] (micro M)	F (arbit units)	delta F (arbit units)
1	0.0000	0.0000	298.0000	0.0000
2	0.5000	0.2880	328.0000	30.0000
3	1.0000	0.5760	360.0000	62.0000
4	1.5000	0.8640	381.0000	83.0000
5	2.0000	1.1520	386.0000	88.0000
6	2.5000	1.4400	390.0000	92.0000
7	3.0000	1.7280	393.0000	95.0000
8	3.5000	2.0160	393.0000	95.0000
9	4.0000	2.3040	394.0000	96.0000
10	5.0000	2.8800	394.0000	96.0000
11	6.0000	3.4500	394.0000	96.0000

PENTASACCHARIDE

	1	2	3	4
Type				
Column Name	microliter of working solution	[pentasac] (micro M)	F (arbit units)	delta F (arbit units)
1	0.0000	0.0000	260.0000	0.0000
2	0.5000	0.0202	277.0000	17.0000
3	1.0000	0.0404	290.0000	30.0000
4	1.5000	0.0606	305.0000	45.0000
5	2.0000	0.0808	314.0000	54.0000
6	2.5000	0.1010	319.0000	59.0000
7	3.0000	0.1212	327.0000	67.0000
8	3.5000	0.1414	331.0000	71.0000
9	4.0000	0.1616	336.0000	76.0000
10	4.5000	0.1818	339.0000	79.0000
11	5.0000	0.2020	339.0000	79.0000
12	6.0000	0.2424	343.0000	83.0000
13	7.0000	0.2828	345.0000	85.0000
14	8.0000	0.3232	346.0000	86.0000
15	9.0000	0.3636	349.0000	89.0000
16	11.0000	0.4444	352.0000	92.0000
17	13.0000	0.5252	352.0000	92.0000
18	15.0000	0.6060	353.0000	93.0000
19	17.0000	0.6868	353.0000	93.0000
20	19.0000	0.7676	353.0000	93.0000
21	21.0000	0.8484	353.0000	93.0000

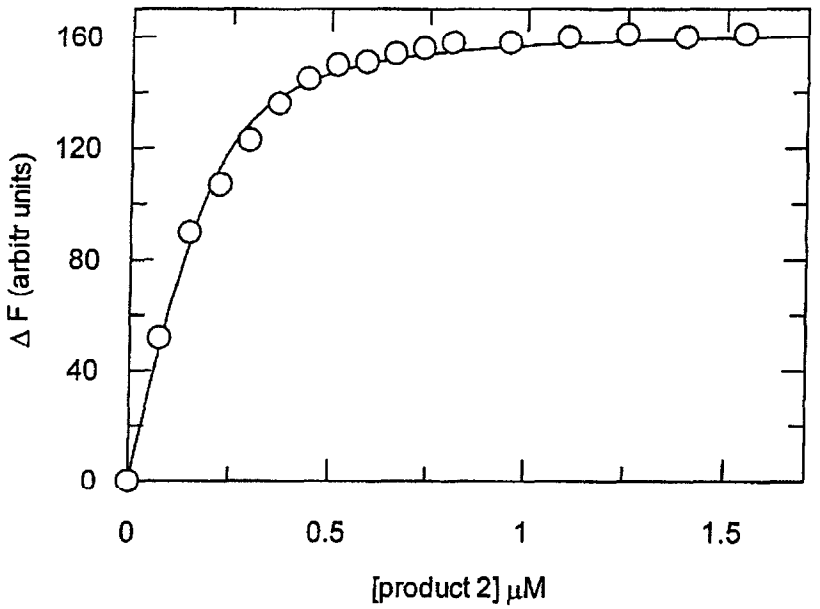
PRODUCT 1



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0012	0.0003
stoichio	0.1863	0.0035

[AT III] = 0.091 μM

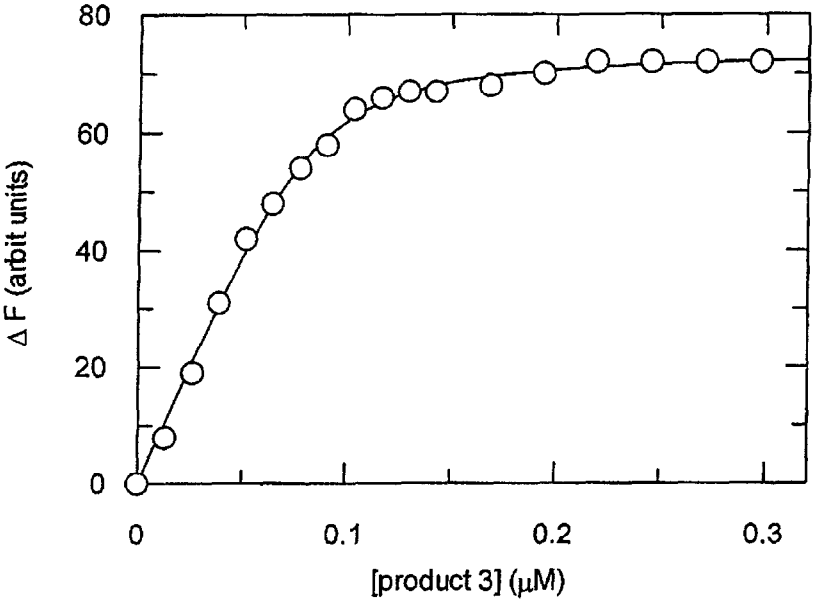
PRODUCT 2



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0339	0.0057
stoichio	0.9093	0.0650

[AT III] = 0.183 μM

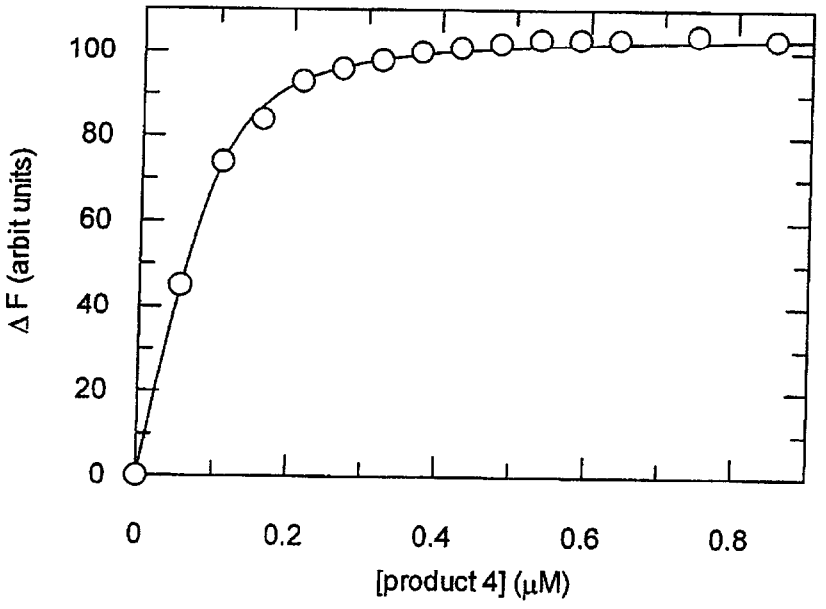
PRODUCT 3



Parameter	Value	Std. Error
equilibrium constant	0.0054	0.0007
stoichio	0.9358	0.0295

[AT III] = 0.078 μM

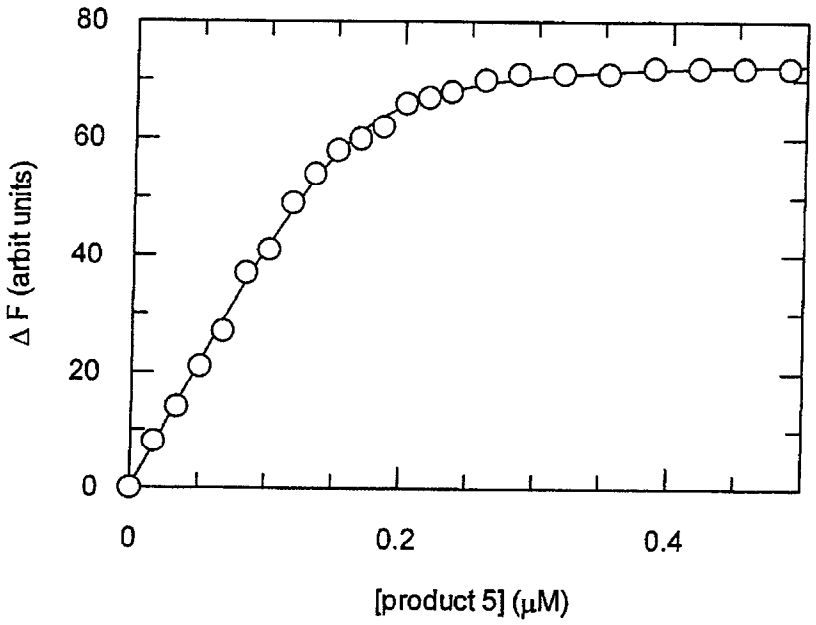
PRODUCT 4



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0148	0.0018
stoichio	0.9260	0.0462

[AT III] = 0.091μM

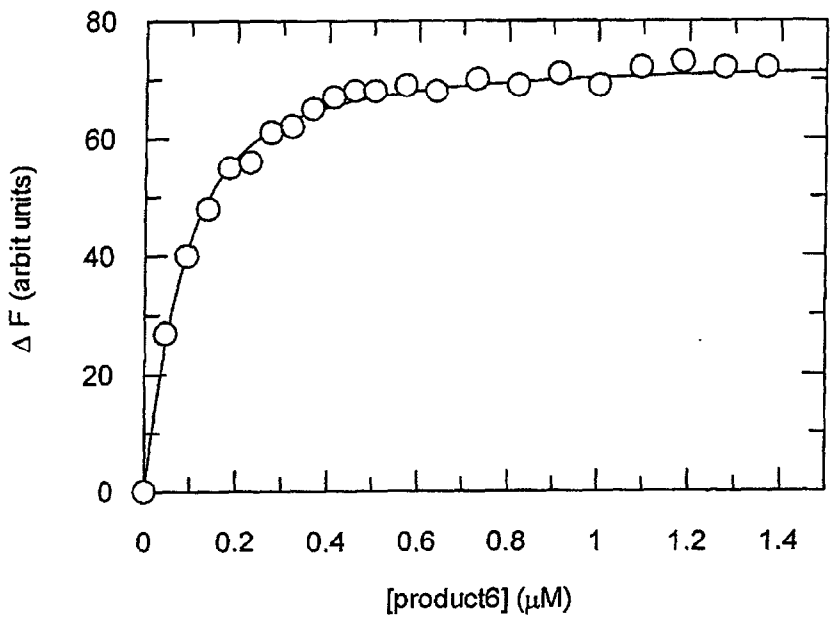
PRODUCT 5



Parameter	Value	Std. Error
equilibrium constant	0.0036	0.0003
stoichio	0.4861	0.0081

[AT III] = 0.078 μM

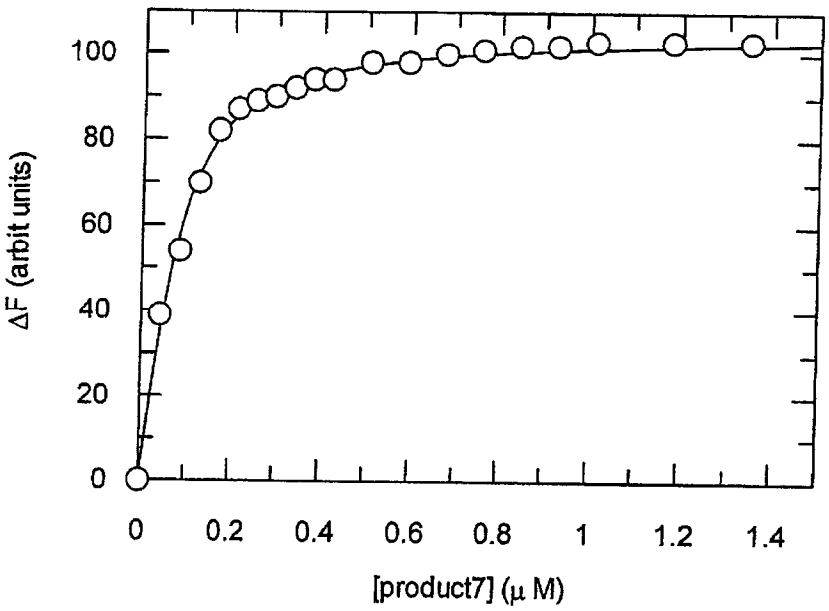
PRODUCT 6



Parameter	Value	Std. Error
equilibrium constant	0.0471	0.0098
stoichio	1.1031	0.1575

[AT III] = 0.078 μM

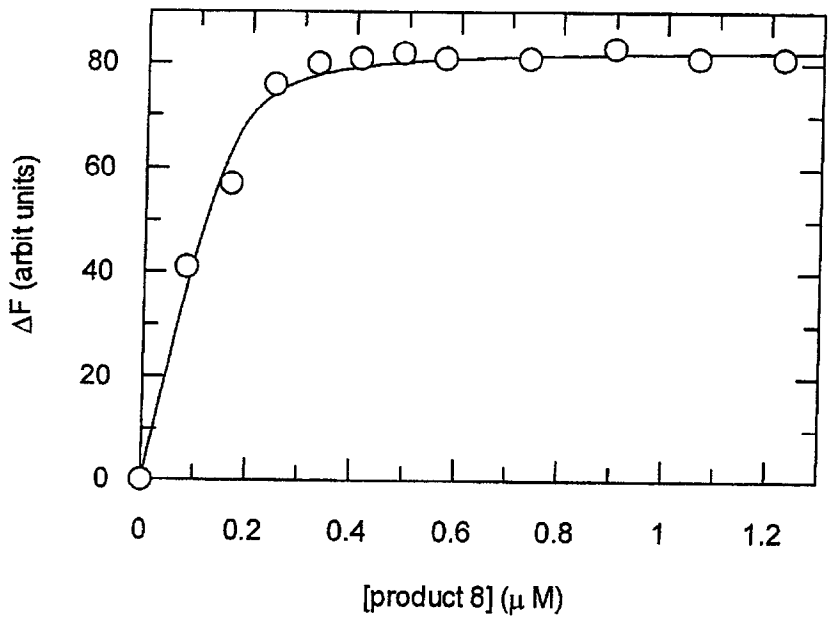
PRODUCT7



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0427	0.0076
stoichio	1.2253	0.1359

[AT III] = 0.091μM

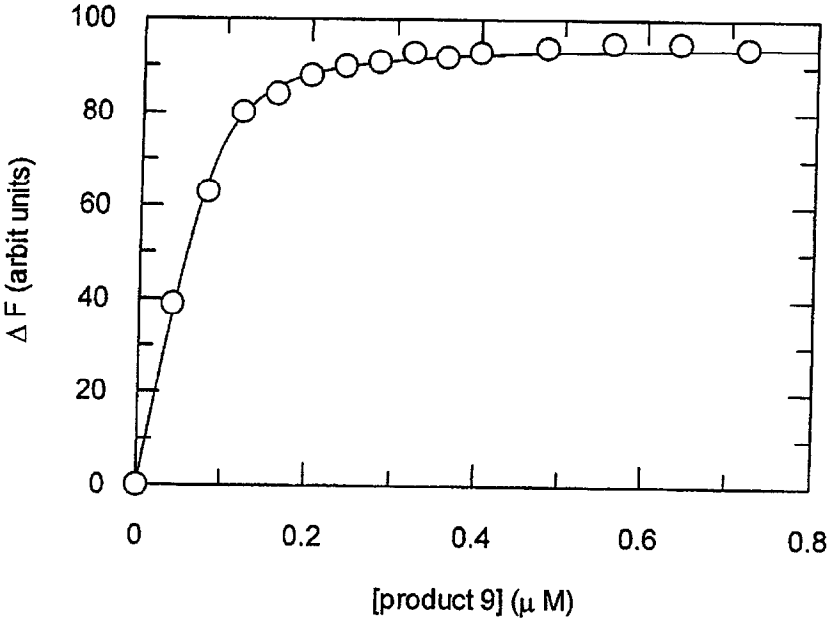
PRODUCT 8



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0129	0.0055
stoichio	1.1049	0.1183

[AT III] = 0.183 μM

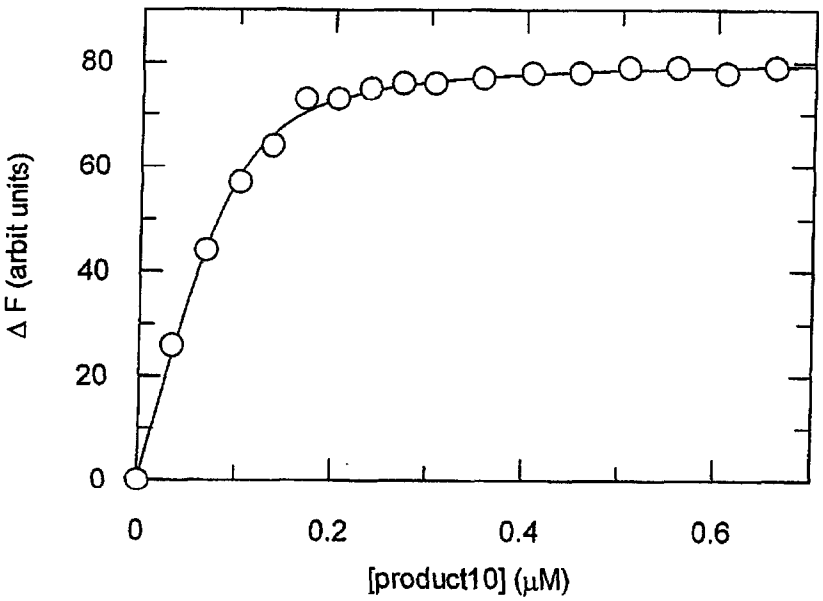
PRODUCT 9



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0094	0.0013
stoichio	1.0387	0.0453

[AT III] = 0.091 μM

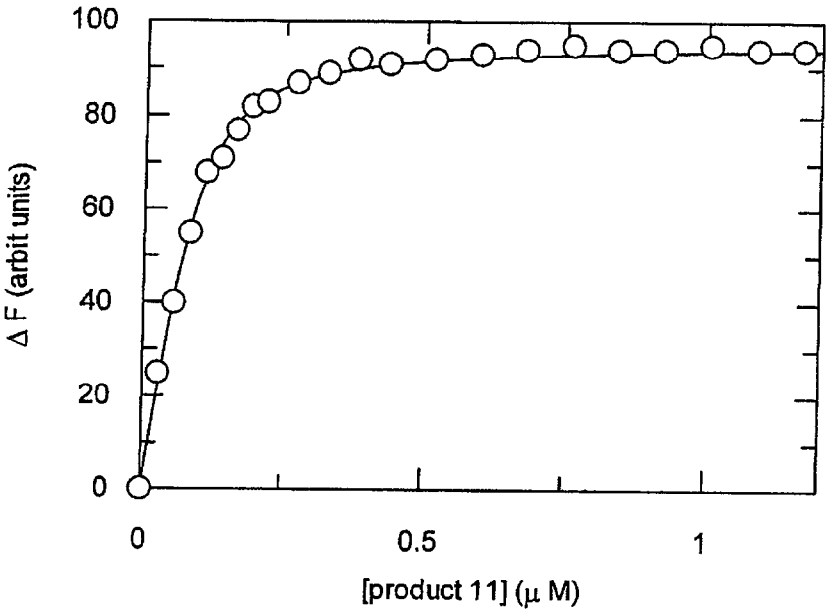
PRODUCT 10



Parameter	Value	Std. Error
equilibrium constant	0.0110	0.0013
stoichio	0.8300	0.0372

[AT III] = 0.078 μM

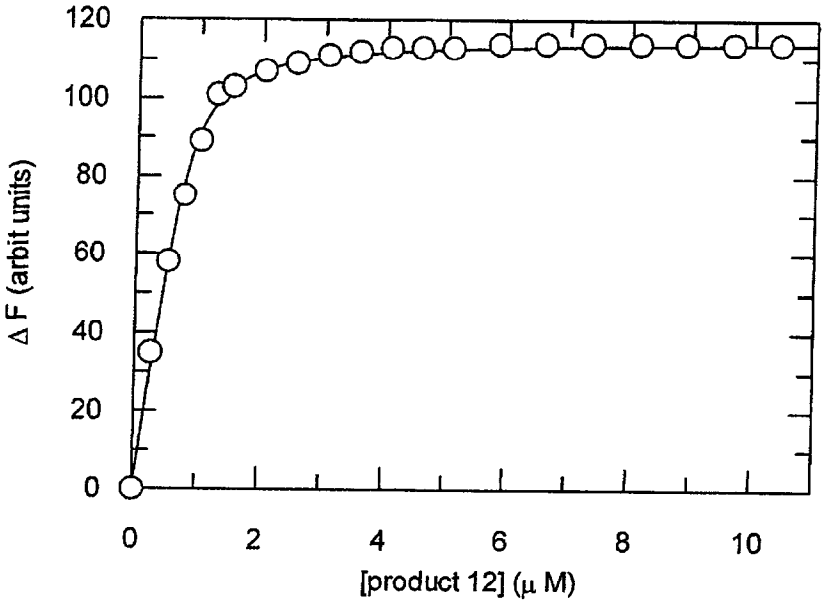
PRODUCT 11



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0177	0.0022
stoichio	0.9588	0.0492

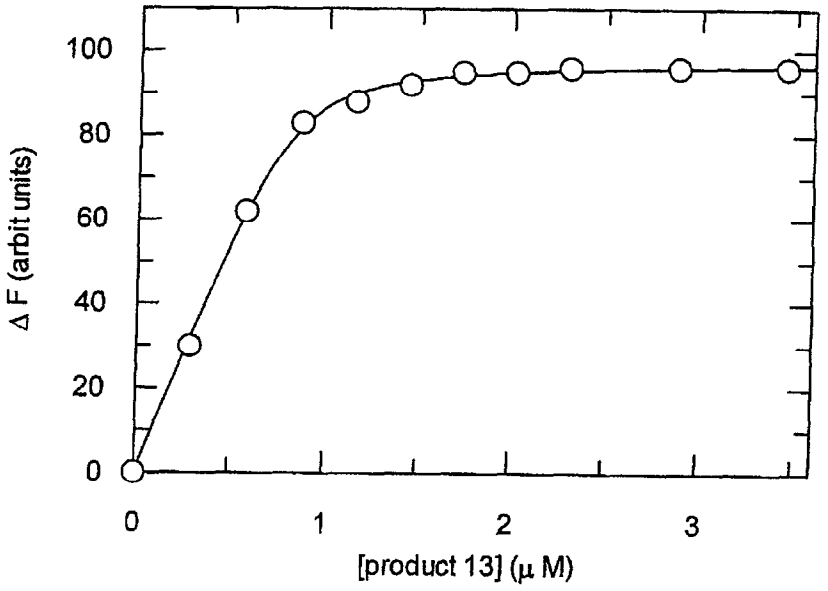
[AT III] = 0.091 μM

PRODUCT 12



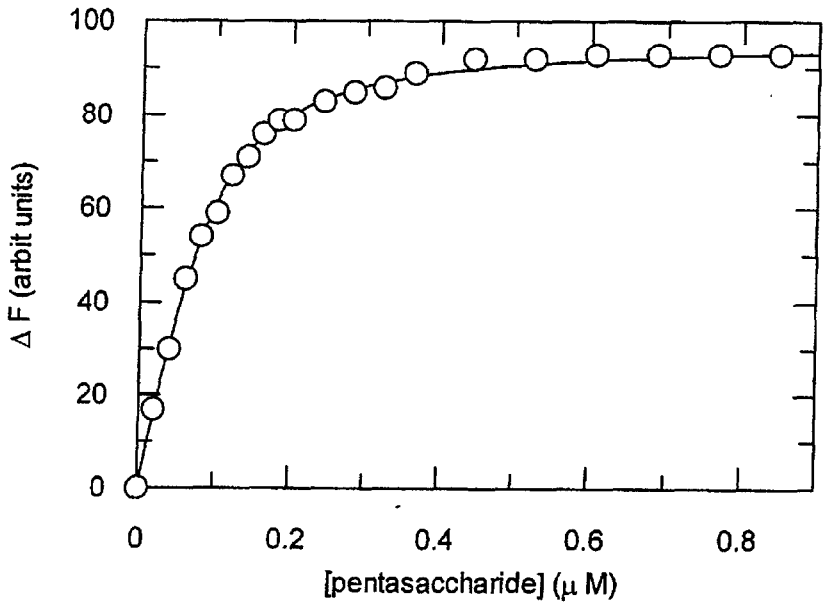
Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0104	0.0014
stoichio	0.1107	0.0046

[AT III] = 0.091 μM



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0036	0.0006
stoichio	0.1103	0.0030

[AT III] = 0.091 μM



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0254	0.0024
stoichio	1.0205	0.0460

[ATIII] = 0.091 μM