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β-Galactosidases of *Escherichia coli* with substitutions for Glu-461 can be activated by nucleophiles and can form β-D-galactosyl adducts

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ABSTRACT

Nucleophiles activated the catalytic actions of β-galactosidases with neutral or positively charged substitutions for Glu-461. Aliphatic carboxylic acids increased the rate of hydrolysis of *o*-nitrophenyl β-D-galactopyranoside if the p*K*a values of the carboxyl groups were $> \sim 3.5$. Amino compounds activated if their p*K*a values were $< \sim 8.5$. Imidazole, azide, and 2-mercaptoethanol also activated. Nucleophiles with high p*K*a values were able to activate the catalysis if the pH was high, and this showed that the lack of activation at pH 7.0 was because of protonation. Kinetic analysis showed that most of the nucleophiles that activated were bound to the active site, since the activation followed Michaelis–Menten type saturation kinetics. The binding seemed to be dependent upon the hydrophobicity; the longer the aliphatic chain, the stronger the binding. Gas–liquid chromatographic analysis showed that adducts of some type were formed during the reactions in the presence of many of the nucleophiles. Three of these adducts were purified and the nucleophiles were found β-linked to D-galactose. This indicates that if an intermediate covalent bond is formed in the mechanism of β-galactosidase action and if the nucleophile reacts to displace it, the intermediate covalent bond must have the α configuration and involve a group other than Glu-461.

INTRODUCTION

Conduritol C *cis*-epoxide irreversibly inactivates β-galactosidase from *Escherichia coli*[1] and reacts almost exclusively with Glu-461, and site-specific amino acid substitutions for Glu-461 cause dramatic reductions in the enzyme activity[2–4]. Kinetic studies by Sinnott and Souchard[5] and by Rosenberg and Kirsh[6] suggest that a group such as Glu-461 could act as a counter ion to aid the formation of a carbocation intermediate from galactose during the reaction, and also that a transient covalent bond could form between such a group and the galactose. If Glu-461 is substituted by a residue that does not have a negative charge, the

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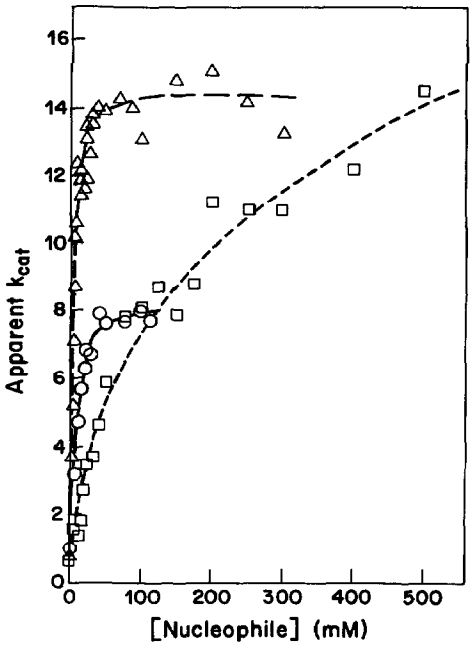

Fig. 1. The apparent k_{cat} values (s^{-1}) plotted vs the concentrations (mM) of three representative nucleophiles; △, sodium azide; ○, hexanoate; □, 6-aminohexanoate.

enzyme is no longer inhibited by 2-amino-2-deoxy-D-galactopyranose[4]. This observation indicates that Glu-461 probably interacts with a positively charged reaction-intermediate. Other work[7] has shown that Glu-461 is probably also an inner-sphere ligand to Mg^{2+} at the active site of β-galactosidase.

Preliminary investigation showed that nucleophilic compounds activated β-galactosidases having substitutions for Glu-461. On the other hand, normal enzyme and many of the enzymes we had in our laboratory, with substitutions at other positions, were not affected. The results of a detailed study of these interactions catalyzed by enzymes with substitutions for Glu-461, especially by Gly, are presented here.

RESULTS

Kinetic analysis of reactions with added nucleophiles.—Several classes of potential nucleophiles were systematically checked for their effects, namely compounds with carboxyl or amine groups, compounds having both amine and carboxyl groups (amino acids), and other groups. Fig. 1 shows plot of the apparent k_{cat} values as functions of the concentrations of three representative nucleophiles (azide, hexanoate, and 6-aminohexanoate). The curves shown are representative of the whole

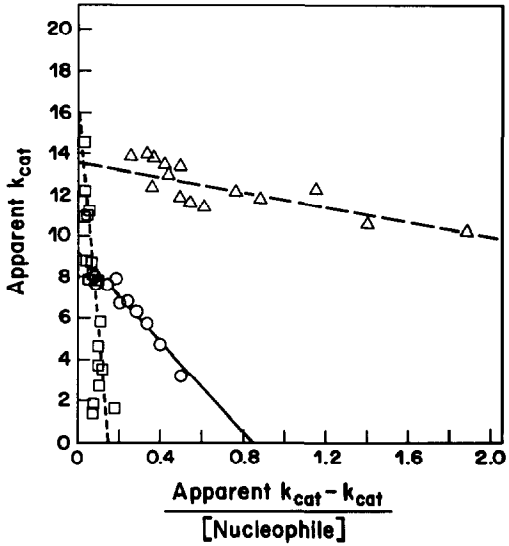

Fig. 2. Plots of the apparent k_{cat} (s^{-1}) {(apparent $k_{cat} - k_{cat}$)/[nucleophile]} (s^{-1} mM^{-1}) for the same three representative nucleophiles as in Fig. 1. The same symbols are used as in Fig. 1.

series of nucleophiles that activated the enzyme. In the absence of nucleophile, the k_{cat} value was very low but, as the nucleophile concentrations were increased, the rates increased to the point of saturation. This is illustrated in another way in Fig. 2 for the same three representative nucleophiles. In this case the apparent k_{cat} was plotted versus {(apparent $k_{cat} - k_{cat}$)/[nucleophile]}. Deschavannes et al.[8] and Huber et al.[9] showed that plots of this type should be linear if an "acceptor" binds to the enzyme and reacts with the galactosyl intermediate. The interaction described here is different from the acceptor interactions described in those papers in that the nucleophiles tested in this study do not react as acceptors for the wild-type enzyme. The kinetics for the two systems should, however, be similar. The intercept of each line on the vertical axis represents the maximum rate at infinite nucleophile concentration, and the slope approximates the dissociation constant of the galactosyl-enzyme–nucleophile complex (analogous to the similar values for Michaelis–Menten kinetics). Note that for the three representative nucleophiles in Fig. 2, there were differences in the slopes and intercepts. Table I gives the pKa values, the intercept values and the slope values for plots of apparent k_{cat} versus {(apparent $k_{cat} - k_{cat}$)/[nucleophile]} of those nucleophiles that we tried that activated E461G-β-galactosidase (the designation, E461G-, indicates that the Glu at position 461 in β-galactosidase from wild-type *E. coli* is replaced by a Gly; other enzymes having substitutions are described in the same manner). In some cases the binding was so poor that the lines were almost vertical. We report these slopes as being "steep" because the values of slopes that one would obtain would be highly inaccurate. A few nucleophiles bound quite well. In

TABLE 1

The pKa values and the values of the slopes (mM) and of intercepts (s^{-1}) of plots of apparent k_{cat} vs {(apparent $k_{cat} - k_{cat}$)/[nucleophile]} [a]

Nucleophile	pKa (s)	Slope	Intercept
Formate	3.77	210	6.8
Acetate [b]	4.76	170	16.9
Propanoate [b]	4.88	150	20.7
Butanoate [b]	4.82	76	14.0
Pentanoate [b]	4.86	31	10.6
Hexanoate [b]	4.88	11	9.2
Isobutanoate [b]	4.86	320	10.6
Benzoate [b]	4.26	58	4.3
Succinate [b]	4.19, 5.48	steep	> 7.5
Maleate [b]	1.92, 6.23	[c]	
β-Alanine	3.60, 10.19	steep	> 1.9
4-Aminobutanoate	4.23, 10.43	1290	19.8
5-Aminopentanoate	4.20, 10.69	250	15.9
6-Aminohexanoate	4.43, 10.75	110	15.9
7-Aminoheptanoate	4.40, 10.70	29	11.6
Hydroxylamine [b]	5.96	steep	> 4.8
2-Aminoethanol	9.50	steep	> 2.4
3-Aminopropanol	9.96	[c]	
2-Bromoethylamine [b]	8.49	57	5.8 [d]
Glycine ethyl ester	7.83	98	20.2
Imidazole [b]	7.05	380	12.5
Azide [b]	4.72	1.8	13.5
2-Mercaptoethanol [b]	9.5	87	4.3

[a] The k_{cat} of the wild-type enzyme is ~540 s^{-1}, while that of E461G-β-galactosidase without nucleophiles is 0.55 s^{-1}. [b] Additions resulted in detectable GLC peaks; the lack of a peak does not indicate that an adduct was not formed, only that it could not be detected. [c] Slight activation. [d] High concentrations caused inhibition; the value of the intercept was obtained with low inhibitor concentrations.

particular, azide had a shallow slope. Some nucleophiles tried * had no effect on activity at 100 mM.

Analyses were performed with wild-type enzyme and also with Y503F-, E537V-, E537Q-, and E537D-β-galactosidases. In none of those cases was there activation with azide. Also, there was no increase in the rate of reaction with the wild-type enzyme and no product was produced which was detectable by GLC. Thus, the effect of the nucleophiles only seems to occur with β-galactosidases having substitutions for Glu-461.

When Glu-461 was substituted with Gln or Lys, rate increases similar to those found with E461G-β-galactosidase were noted with azide or 2-mercaptoethanol.

* Compounds tried (100 mM) that did not activate at pH 7.0 (pKa values are listed in brackets): glycine (2.34, 9.6); ammonia (5.98); methylamine (10.64); ethylamine (10.67); propylamine (10.58); butylamine (10.68); 1,3 propylamine (8.64, 10.62); iodoacetate (3.12); chloroacetate (2.86); trichloroacetate (0.65); D-gluconate (3.86); phosphate (7.21); arsenate (7.0); fluoride (3.2); chloride (−5.7); bromide (−7.7); iodide (−10.7); pyridine (5.14); cyanide (9.4); phenol (9.95).

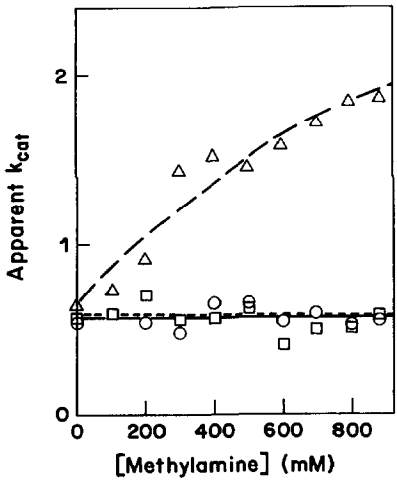

Fig. 3. Plots of apparent k_{cat} (s^{-1}) vs the concentration of methylamine (mM) at three different pH values: ○, 7.0; □, 8.5; △, 9.5.

Those were the only nucleophiles tested, but it is likely that if all of the nucleophiles tested with E461G-β-galactosidase would have been investigated, similar results would have been obtained. When Asp was substituted for Glu-461, there was no detectable rate increase. When Glu-461 was substituted by His, a small activation was found (~ 2-fold).

2-Bromoethylamine has an amino group with a relatively low pKa. It did activate the enzyme. However, it inhibited the reaction at high concentrations.

Effect of pH.—At pH 8.5, the intercept of an apparent k_{cat} vs {(apparent $k_{cat} - k_{cat}$)/[nucleophile]} plot was 31 s^{-1} for 2-mercaptoethanol (compared to 4.3 s^{-1} at pH 7.0) and 28 s^{-1} for 2-aminoethanol (compared to > 2.4 s^{-1} at pH 7.0). Fig. 3 shows that methylamine did not activate at all at either pH 7.0 or 8.5 but it significantly activated the reaction at pH 9.5.

Nucleophiles that form products.—Table I also indicates whether or not a definite GLC peak could be detected after incubation (for those compounds that activated the reaction).

Purification of adducts.—The purified products of the incubations of imidazole, of 2-mercaptoethanol, and of azide with E461G-β-galactosidase and *o*-nitrophenyl β-D-galactopyranoside were white powders. Each was pure by GLC. Attempts were made to purify the acetate adduct, but it was found to be very unstable, after elution through a G-10 column mainly galactose and acetate were found.

NMR identification.—*N-(β-D-Galactopyranosyl)imidazole*: ^{1}H NMR: δ 5.18 (d, 1 H, $J_{1,2}$ 8.8 Hz, H-1), 3.80 (m, 2 H, H-2,4), 3.40–3.48 (m, 3 H, H-3,6), 3.60 (m, 1 H, H-5), 8.27 (s, 1 H, *im*H-2′), 7.16 (s, 1 H, *im*-H-4′), 7.46 (s, 1 H, *im*H-5′); ^{13}C NMR: ∂ 90.0 (1 C, C-1), 73.8 (1 C, C-2), 77.6 (1 C, C-3), 72.2 (1 C, C-4), 81.5 (1 C, C-5), 63.6 (1 C, C-6), 139.9 (1 C, *im*C-2′), 129.1 (1 C, *im*C-4′), 122.0 (1 C, *im*C-5′). *β-D-Galactopyranosyl azide*: ^{1}H NMR: δ 4.66 (d, 1 H, $J_{1,2}$ 8.6 Hz, H-1), 3.51 (dd, 1

H, $J_{2,3}$ 9.7 Hz, H-2), 3.68 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 3.95 (d, 1 H, $J_{4,5} \sim 0$, H-4), 3.74–3.78 (m, 3 H, H-5,6); ^{13}C NMR: ∂ 91.4 (1 C, C-1), 71.2 (1 C, C-2), 73.5 (1 C, C-3), 69.5 (1 C, C-4), 78.5 (1 C, C-5), 61.8 (1 C, C-6). *2-(β-D-Galactopyranosylthio)ethanol*: ^{1}H NMR: δ 4.49 (d, 1 H, $J_{1,2}$ 9.8 Hz, H-1), 3.56 (dd, 1 H, $J_{2,3}$ 9.2 Hz, H-2), 3.65 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 3.97 (d, 1 H, $J_{4,5} \sim 0$, H-4), 3.70–3.76 (m, 3 H, H-5,6), 2.87, 2.95 (dt, 2 H, $J_{1',2'}$ 6.7, J_{vic} 14.0 Hz, *et*H-1′), 3.79 (dd, 2 H, *et*H-2′); ^{13}C NMR: δ 86.2 (1 C, C-1), 70.2 (1 C, C-2), 74.5 (1 C, C-3), 69.4 (1 C, C-4), 79.5 (1 C, C-5), 61.9 (1 C, C-6), 32.9 (1 C, *et*C-1′), 61.9 (1 C, *et*C-2′). (The *im* and the *et* and the prime superscripts designate the imidazole and the ethane group protons and carbons.) The chemical shifts and the coupling constants (especially the $J_{1,2}$ values of ~ 9.0 Hz) clearly identify the adducts as being of the β configuration. There was no hint of any α product. For the 2-mercaptoethanol adduct, the non-equivalence of the proton shifts on the carbon adjacent to sulfur show that the glycosidic bond was to sulfur and not to oxygen.

DISCUSSION

The activation of β-galactosidases having substitutions for Glu-461 by nucleophiles and the formation of adducts entices one to reason that the added nucleophiles are simply playing the role of the missing side-chain of Glu-461. This is especially tempting since nucleophiles having carboxyl groups activated strongly and Glu-461 presumably carries out its function via its carboxyl group. However, the fact that the nucleophiles formed β-D-galactosyl adducts indicates that the nucleophile is not directly replacing the aglycon. If the replacement were direct, α-linked adducts should have been obtained. A probable reason that β adducts are formed is that a nucleophile on the enzyme displaces the aglycon of the substrate and forms an α bond to the galactose, and the added nucleophiles then displace the galactose to form β-linked adducts. The nucleophile on the β-galactosidase that forms an α bond cannot be Glu-461 (because that Glu is absent in E461G-β-galactosidase) but it could be Glu-537. Gebler et al.[10] have recently reported that 2-deoxy-2-fluoro-D-galactopyranose becomes covalently bound to Glu-537 of β-galactosidase (*E. coli*) when 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside is the substrate, and Yuan et al.[11] have shown by site-specific studies that Glu-537 is essential for activity.

Glu-461 must play some role at the active site. No activation occurred and no adducts were formed when the nucleophiles that activated E461G-β-galactosidase were added to enzyme from wild-type *E. coli*. The presence of Glu-461 must in some way prevent the reaction with the nucleophile. Glu-461 is necessary for the binding of analogues of cationic glycon such as protonated 2-amino-2-deoxy-D-galactopyranose[4], and the residue is important for binding Mg^{2+} (ref 7). Also, when Glu-461 is replaced by His, there is good evidence that the His forms a quasi-stable covalent bond with galactose[4] (also unpublished observations from our laboratory). Glu-461 must be close to the anomeric carbon of the intermediate that

forms and it must aid in its formation. Also, it must be poised in such a way as to prevent nucleophiles form reacting (possibly by the juxtaposition of its negative charge).

The reactivities of the nucleophiles studied provide information about the nature of the interaction. In general, nucleophiles that have pKa values between ~ 3.5 and ~ 8.5 reacted with the galactosyl enzyme and increased the reaction rate of E461G-β-galactosidase with o-nitrophenyl β-D-galactopyranoside. Compounds having pKa values < 3.5 are probably not nucleophilic enough to react, or the adducts decompose after they are formed, because compounds having low pKa values are good leaving groups. The acetate adduct was definitely unstable, and its instability supports the latter possibility. With formate and succinate, no adduct could be detected. Since the reaction rates were increased by significant amounts in the presence of these two compounds, it may be that the adducts were formed but were very unstable and broke down rapidly. In cases where the pKa was < 3.5, the compounds did not speed up the reaction at all and most likely, in those cases, the compounds did not react at all because of their poor nucleophilicity.

The most probable reason that some of the compounds having high pKa values did not react was that such compounds are protonated at pH 7.0. The pH experiments strongly support this reasoning. As the pH of the reaction was increased, the ability of mercaptoethanol, 2-aminoethanol, and methylamine to act as nucleophiles increased dramatically. Some compounds did react, even though their pKa values were > 8.5. 2-Aminoethanol (pKa 9.5) was somewhat reactive at pH 7.0 and 3-aminopropanol (pKa nearly 10) reacted (albeit poorly). It is possible that the hydroxyl groups position the amino groups for reaction in these two cases. 2-Mercaptoethanol was also an exception. Sulfhydryl groups are very good nucleophiles and the reaction is probably able to proceed slowly at pH 7.0, even with only very small amounts of the unprotonated nucleophile. Also, the protonated form of the sulfhydryl group may be a good enough nucleophile to activate the enzyme.

It is instructive to look at how the nucleophiles of a series increased the rate at which o-nitrophenyl β-D-galactopyranose was hydrolyzed by E461G-β-galactosidase. In the case of the carboxylic acids, formate bound quite poorly (as indicated by the high value of its slope) but was quite a good activator. As the chain length of the acids become longer, there was a constant increase in binding ability (lowering of the slope value). Presumably, the "binding site" has hydrophobic properties and the binding increases as the length of the hydrocarbon chain increases. Isobutanoate was an exception. Its bulk and shape probably decrease its binding capability. With the aliphatic series, the reactivity increased to a maximum with propanoate and then decreased with the longer acids. As the pKa values of all of the acids larger than acetic acid are about the same, the reactivity must be associated with the positioning. The binding of the C_3 acid (propanoate) must be such that the position of the carboxyl group is optimal. Benzoate bound well, but was not very reactive. Its pKa is somewhat lower than that of the more reactive

carboxylic acids and, also, positioning may be important. The activation by amino acids followed a similar progression to that of the aliphatic acids. As the p*Ka* values of the amino groups of the amino acids are high, the carboxyl components must have been the reactive nucleophiles at pH 7.0. If one compares the slopes (Table I) for the amino acids with the aliphatic acids of equal carbon number, the binding was poorer (slope was steeper) for the amino acid (β-alanine vs propionoate; 4-aminobutanoate vs butanoate, etc.). Thus, positively charged amino groups must inhibit binding.

It is not clear why adducts produced by the reactions with the amino acids could not be detected by the gas chromatography technique used. Possibly, as seems to have been the case with formate and succinate, the adducts may have been too labile to allow detection. It was also found that no product was detected when glycine ethyl ester was the added nucleophile. The reactive component in that case must have been the amino group. Again, it is possible that it was very labile and decomposed before or during the derivatization[12] that is necessary for gas chromatographic analysis.

With azide as the nucleophile, a shallow slope was obtained. Azide is a small molecule with some positive character, but one net delocalized negative charge. It may bind readily because of local complementary features at the active site.

It is possible that 2-bromoethylamine causes an adduct to form and that the adduct with bromine then becomes an affinity reagent. A nucleophile of some sort on the enzyme could displace bromine and form a covalent bond to inactivate the enzyme at high concentrations. In essence, this would be a mechanism-based inactivator (suicide inhibitor). Further studies on this are needed.

These reactions have significant potential as a means of readily producing β-D-galactosyl adducts without the necessity of protecting the other hydroxyl groups. In addition, it is also possible that the unstable acetyl adducts that form could be utilized to produce α-galactosyl derivatives by direct substitution. Studies to investigate the utility of this are underway.

EXPERIMENTAL

Chemicals.—The *o*-nitrophenyl β-D-galactopyranoside and TES buffer were obtained from Sigma or Boehringer-Mannheim. Most of the nucleophiles used in the study were from Aldrich. Other chemicals were from Fisher or equivalent suppliers. In each case the chemicals used were the purest grades available.

Enzyme production and purification.—β-Galactosidases with substitutions for Glu-461 were produced by using site-specific mutagenesis as described by Cupples et al.[4]. Growth of the cells and the purification procedures followed were also described by Cupples et al.[4]. The enzymes were analyzed by SDS PAGE (8–25% acrylamide) and were > 96% pure.

Assays.—TES assay buffer (30 mM, pH 7.0) with NaCl (140 mM) and $MgSO_4$ (1 mM) was used. Tubes containing assay solution were pre-incubated in a 25°C

water-bath for 5 min before addition of enzyme. After the enzyme was added, the absorbance at 420 nm was monitored (25°C) as a function of time. The absorbance changes observed were converted to units/mg (1 unit is 1 μmol of *o*-nitrophenol per min). The concentration of nucleophile added depended upon the nucleophile. For kinetic analysis, *o*-nitrophenyl β-D-galactopyranoside [at a highly saturating level (2 mM) since the K_m of E461G-β-galactosidase for *o*-nitrophenyl β-D-galactopyranoside is 8 μM] was added and the rates as functions of nucleophile concentration were followed. These rates were considered to be equivalent to V_m values and experiments were carried out that confirmed this. The V_m values were converted to k_{cat} values and apparent k_{cat} values.

Formation and isolation of products.—The presence of adducts produced as a result of adding nucleophiles to a mixture with *o*-nitrophenyl β-D-galactopyranoside and E461G-β-galactosidase was tested for by GLC methods previously described[12]. Assays were made with and without the nucleophile and the presence of new peaks was looked for.

For the isolation of the adduct, 1 μg of the enzyme was added to 100 mL of a solution with 500 mM nucleophile, 20 mM *o*-nitrophenyl β-D-galactopyranoside, 145 mM NaCl, and 1 mM $MgSO_4$ at pH 7.0. Buffer was not added, since the buffering components we tried either acted as nucleophiles or the buffer components were difficult to remove. The pH actually did not change to any significant extent during the reaction. The mixture was incubated overnight. The flask was then heated for 3 min to 60°C in order to inactivate the enzyme and halt the activity, and the mixture was cooled in ice. The solution was lyophilized and the mixture was eluted with water through a glass columm (1 × 150 cm) containing Sephadex G-10, and the fractions were analyzed by GLC. Fractions containing the putative galactosyl-adduct were pooled, concentrated by lyophilization, and then eluted through the column again. At this point the products were pure and ready for analysis.

NMR identification of products.—The purified products were dissolved in D_2O and lyophilized (twice). NMR spectra were recorded on a Bruker AMX-500 spectrometer operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. The spectra were acquired at 27°C using a 5-mm probe. One-dimensional proton spectra were acquired in D_2O or Me_2SO with pre-saturation of the residual HOD resonance at 4.8 ppm (for D_2O samples) and were referenced to internal sodium 4,4-dimethyl-4-silapentanesulfonate (0 ppm). One-dimensional ^{13}C spectra were acquired with composite pulse proton decoupling. Two-dimensional HMQC with decoupling of ^{13}C, and double-quantum-filtered COSY NMR spectra were acquired using standard Bruker pulse programs. The data obtained in these various ways were used to assign the signals.

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