Gas Chromatography and Mass Spectrometry of Trimethylsilyl Sugar Phosphates[1]

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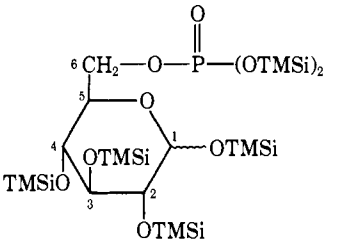
Abstract: The low-resolution mass spectra of completely trimethylsilylated α-D-glucopyranose 6-phosphate, β-D-ribofuranose 5-phosphate, β-D-fructofuranose 6-phosphate, β-D-fructofuranose 1-phosphate, and 6-phosphogluconate obtained by combined gas chromatography–mass spectrometry are described. The electron beam induced fragmentation of these substances is discussed with supporting data obtained by labeling with trimethylsilyl-d_9 groups as well as by the use of carbon chain deuterated D-glucose 6-phosphate and D-fructose 6-phosphate. The mass spectra of trimethylsilyl-α-D-galactopyranose and -galactofuranose 6-phosphates are compared with special reference to the mass spectral differences between furanose and pyranose forms of phosphorylated carbohydrates. The various carbohydrate types studied—aldohexose phosphate, ketohexose 1- and 6-phosphate, aldopentose phosphate, and phosphohexonate—are shown to undergo fragmentations which in many cases can be related to their structures. In several instances fragment ions have been shown to contain specific carbon atoms of the carbohydrate. Several phosphorus containing ions, some highly rearranged and some bearing specific carbon atoms of the parent sugar, are observed. The most interesting phosphorus containing rearrangement ion is *m/e* 387 which is shown to be tetrakis(trimethylsilyl)phosphate. A summary of ions of particular use for the identification of carbohydrate structural types and assignment of specific carbon and hydrogen atoms to fragment ions is presented.

One of the most important recent analytical developments in the field of carbohydrate chemistry has been application of the trimethylsilyl (TMSi) derivative to the gas chromatography of carbohydrates and other polyhydroxy compounds.[3] The broad usefulness of this excellent separatory technique has recently been reviewed.[4] With the advent of combined gas chromatography–mass spectrometry the TMSi derivative has become of even greater importance and interest. The high degree of volatility and thermal stability which the TMSi derivative confers on polyols of all types makes a wide range of substances, including many of considerable biological interest, potential candidates for mass spectrometry *via* the gas chromatographic inlet.

The mass spectrometry of TMSi ethers was first reported by Sharkey, *et al.*[5] Subsequently a number of investigations have been carried out describing the mass spectrometry of a variety of compounds. This literature is briefly reviewed in the recent investigation by DeJongh, *et al.*,[6] of the gas chromatography and mass spectrometry of nonphosphorylated TMSi carbohydrates.

Results and Discussion

The Mass Spectrum of Hexa-*O*-trimethylsilyl-α-D-glucopyranose 6-Phosphate (1). The bar graph presentation of the mass spectrum of **1** is shown in Figure 1. Note that, rather than present the spectrum relative to *m/e* 73 (the most abundant ion), the base peak was chosen to be the next most abundant ion, *m/e* 204. This was done in the interest of a more useful visual presentation, *m/e* 73 being up to 300% of the next most abundant species in the compounds studied. The tabulated data for the spectrum of **1** are found in Table I. It should be noted that among the four spectra presented in Table I, three different base peaks have been chosen. As an aid in the interpretation of the spectrum of **1**, hexa-*O*-TMSi-α-D-glucose 6-phosphate-6,6-d_2 (**2**), hexa-*O*-TMSi-α-D-glucose 6-phosphate-3,4,5,6,6-d_5 (**3**), hexa-*O*-TMSi-α-D-glucose 6-phosphate-1,2,3,4,5,6,6-d_7 (**4**), and hexa-*O*-TMSi-d_9-α-D-glucose 6-phosphate (**5**) were also examined by combined gas chromatography–mass spectrometry. The mass shifts, relative to unlabeled **1**, of selected ions from these spectra are given in Table II.



1

2, 6, 6-d_2
3, 3, 4, 5, 6, 6-d_5
4, 1, 2, 3, 4, 5, 6, 6-d_7
5, TMSi = $(CD_3)_3Si$

The molecular ions of **1** and the deuterated species of **1** are found in trace amounts. In Scheme I are shown several ions formed from $M\cdot^+$ by the loss of $CH_3\cdot$, TMSiOH, and bis(TMSi)phosphoric acid. There are no metastable ions to support this scheme but the labeling patterns found in Table II are consistent with the proposed structures.

Another sequence which may retain an intact pyranose ring terminates with *m/e* 271. The presence of *m/e* 603 in the spectrum is obscured by the natural

(1) Presented in part at the 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1968, Abstract C-35.
(2) To whom requests for reprints should be sent.
(3) C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
(4) "Silylation of Organic Compounds," A. E. Pierce, Pierce Chemical Co., Rockford, Ill., 1968.
(5) A. G. Sharkey, R. A. Friedel, and S. H. Langer, *Anal. Chem.*, **29**, 770 (1957).
(6) D. C. DeJongh, T. Radford, J. D. Hribar, S. Hannessian, M. Bieber, G. Dawson, and C. C. Sweeley, *J. Am. Chem. Soc.*, **91**, 1728 (1969).

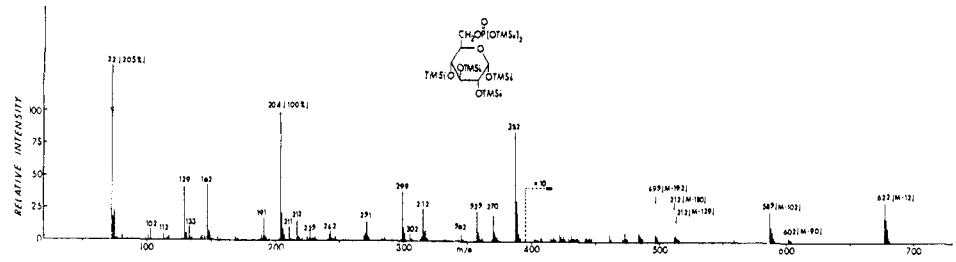
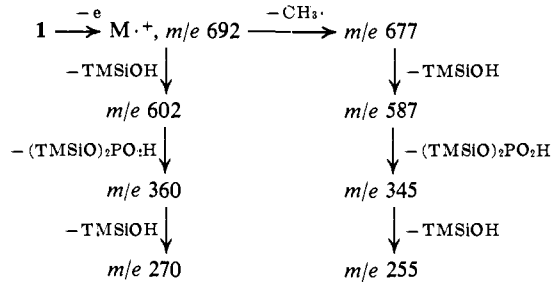


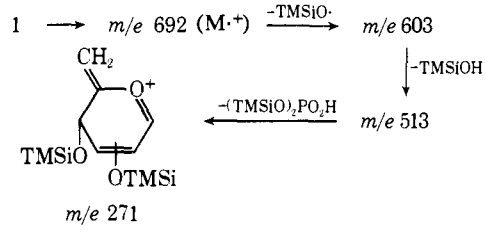
Figure 1. The 70-eV mass spectrum of hexakis(trimethylsilyl)-α-D-glucopyranose 6-phosphate (**1**), mol wt 692.

heavy isotope A + 1 peak of *m/e* 602. The calculated abundance of A + 1 relative to *m/e* 602 is 53% while 80% is observed; the balance is presumably *m/e* 603

Scheme I



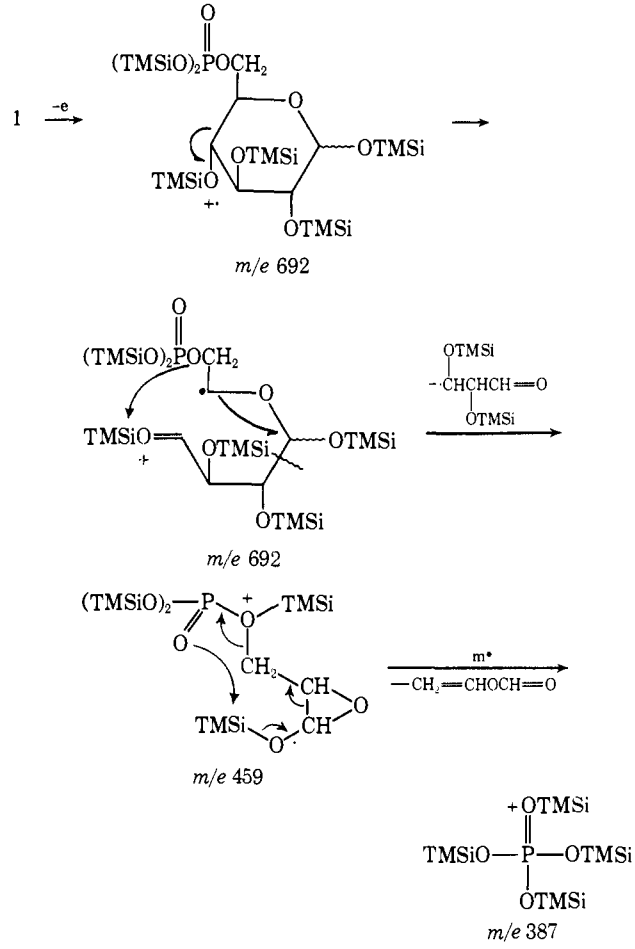
formed as shown. This process also takes place with TMSi-fructose 6-phosphate (**7**), as it does with TMSi-ribose 5-phosphate (**6**). In the latter case, the ion



analogous to *m/e* 271 is found at *m/e* 169. These ions appear to owe their high abundance to the facile elimination of bis(TMSi)phosphate since they are minor ions in nonphosphorylated sugars.[6]

Of particular interest in the spectrum of **1** are the ions which retain the phosphorus atom: *m/e* 387, 370, 357, 343, 315, 314, 299, 227, and 211. These fragments carry a large portion of the total ion current in the spectra of the TMSi-phosphorylated sugars which we have examined.

Of the phosphate ions observed, the most remarkable is *m/e* 387 which has the structure $(TMSiO)_4P^+$ or $(TMSiO)_3P{=}O^+TMSi$. Table II shows, from labeling with $(CD_3)_3Si$- groups (mass spectrum of **5**), that the ion carries four TMSi groups and that glucose labeled on the carbon chain (**2**, **3**, and **4**) introduces no label into *m/e* 387. A metastable ion at *m/e* 326.5 relates the trace abundance ion *m/e* 459 to *m/e* 387 (calculated m* 326.3). This metastable ion is found in each of the TMSi sugar phosphates. The mass spectrum of tris-(TMSi)phosphate has been examined for *m/e* 387[7] to determine if this ion is formed from the abundant $TMSi^+$ ion (*m/e* 73) by an ion-molecule reaction in the spectrometer. Since none was found it seems that *m/e* 387 must arise from an intramolecular rearrangement, in the manner of ions such as 147, 191, etc. The ability of TMSi groups to undergo migration in the mass spectrometer is well known;[5-10] however, the high abundance of *m/e* 387, which is formed by the rearrangement of two TMSi groups, is most surprising. The observation of the metastable ion suggests that the rearrangement is stepwise, *m/e* 459 being an intermediate. While *m/e* 459 is a minor component of the spectrum of **1** it is present in the spectrum of TMSi-6-phosphogluconate (**8**) in greater abundance. The mass shift of this ion to *m/e* 495 in the spectrum of TMSi-d_9-6-phosphogluconate is consistent with the structure given. A possible route to *m/e* 387 is shown.



(7) M. Zinbo and W. R. Sherman, *Tetrahedron Lett.*, **33**, 2811 (1969).

(8) J. Diekman, J. B. Thomson, and C. Djerassi, *J. Org. Chem.*, **32**, 3904 (1967).

(9) J. A. McCloskey, R. N. Stillwell, and A. M. Lawson, *Anal. Chem.*, **40**, 233 (1968).

(10) J. A. McCloskey, A. M. Lawson, K. Tsuboyama, P. M. Krueger, and R. N. Stillwell, *J. Am. Chem. Soc.*, **90**, 4182 (1968).

Table I. Mass Spectra of Sugar Phosphates[a]

m/e	TMSi-α-D-Glucose 6-phosphate (1)	TMSi-β-D-Ribose 5-phosphate (6)	TMSi-β-D-Fructose 6-phosphate (7)	TMSi-6-Phosphogluconate (8)	m/e	TMSi-α-D-Glucose 6-phosphate (1)	TMSi-β-D-Ribose 5-phosphate (6)	TMSi-β-D-Fructose 6-phosphate (7)	TMSi-6-Phosphogluconate (8)
73	261	187	222	323	343	2	4	5	1
75	21	18	19	27	345	6	1	6	1
81	4	2	2	1	348	0.5	0.5	-	0[c]
101	5	3	4	14	356	1	2	2	1
103	10	5	11	9	357	25	2	2	42
113	5	2	4	3	360	2	-	1	3
116	5	2	1	16	361	2	-	3	3
129	42	17	21	22	369	2	2	3	3
131	7	4		10	370	15	-	-	7
133	12	13	125	17	371	-[c]	5	1	-
143	7	10	8	9	373	3	2	3	3
145	3	8	4	6	387	76	3	17	82
147	53	39	61	86	423	t[c]	0[c]	t[c]	2
157	4	3	5	12	445	0.3	0.2	0.5	0
169	3	29	3	4	459	t	t[c]	1	5
177	t	4	3	1	471	1	t	0[c]	13
189	4	2	2	8	513	0.5	0	0.5	0
191	17	9	6	8	561	t	t	t	2
204	100	4	2	14					
211	10	17	13	9	M − 255[b]	437	335	437	525
215	1	37	20	3		t	t	t	t[c]
217	16	27	18	34		497	395	497	585
221	2	1	2	8	M − 195	1	t	t	1
225	3	4	4	4		512	410	512	600
227	8	11	12	5	M − 180	t	t	1	t
230	2	53	40	6		513	411	513	601
243	7	16	5	5	M − 179	t	t	t	t
247	5	0.3	2	9		587	485	587	675
255	1	0.5	1	-[c]	M − 105	1	1	t	t
257	1	2	9	1		589	487	589	677
258	1	5	-[c]	1	M − 103	t	t	41	1
259	1	7	-	5		602	500	602	690
269	3	2	3	3	M − 90	t	t	t	0[c]
270	5	-[c]	3	-		603	501	603	691
271	12	0.3	14	3	M − 89	t	t	t	0
299	46	52	55	100		677	575	677	765
305	6	2	3	7	M − 15	2.4	5	5	6
314	3.4	2.8	4.6	8.9		692	590	692	780
315	31	100	100	52	$M^{\cdot+}$	t	t	t	-
328	1	10	4	3					
331	1	1	2	3					
332	1	0.4	0.7	2					
333	1	1	1	26					

[a] The precision of measurement is ±10% absolute (standard deviation) of all ions of greater than 5% abundance. Intensity data are the abundance of the ion relative to the base peak. No correction for natural isotope contribution is made. All sugars are completely trimethylsilylated, *i.e.*, ethers and esters of trimethylsilanol. [b] Ions in the lower portion of the table are entered as fragment ions minus neutral fragments. The upper entry is the *m/e* of each ion, the lower entry is the abundance. The neutral fragments lost are as follows: 15, $CH_3\cdot$; 89, $TMSiO\cdot$; 90, TMSiOH; 103, $TMSiOCH_2\cdot$; 105, TMSiOH + $CH_3\cdot$; 179, TMSiOH + $TMSiO\cdot$; 180, 2TMSiOH; 195, 2TMSiOH + $CH_3\cdot$; 255, $O{=}P\text{-}(OTMSi)_2OCH_2\cdot$. [c] -, not measured because of interfering peak; 0, none observed; t, less than 0.5%.

Another phosphorus-containing ion is *m/e* 357. Deuterium labeling (Table II) supports the structure given below and indicates that 357 retains C-5 and C-6 of the glucose chain. Consistent with this is the fact that the spectrum of **3** also shows this ion to be shifted by three mass units. Since **1** is in the pyranoside form one TMSi group must migrate, perhaps to the ring oxygen of the pyranoside.

m/e 357

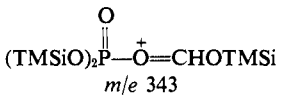
The ion *m/e* 343 is the only clear example of the migration of a TMSi phosphate moiety which we have found. This ion has enhanced abundance in phosphorylated sugars in the furanose form which is most striking in the comparison of the spectra of TMSi-α-D-galactofuranose 6-phosphate and TMSi-α-D-galactopyranose 6-phosphate (Table III). TMSi-ribose 5-phosphate (**6**) and TMSi-fructose 6-phosphate (**7**) also produce this ion in the spectrometer to a degree somewhat greater than do **1** and TMSi-6-phosphogluconic acid. The ion from **7** is sufficiently abundant to observe its mass shift when deuterium labeled. Using β-D-fructose 6-phosphate-3,4,5,6,6-d_5 and -6,6-d_2 we have observed their TMSi derivatives to give spectra wherein *m/e* 343 is shifted to *m/e* 344 with the d_5 fructose but to undergo no shift with the d_2 fructose. These results show that C-1, -2, and -6 are not retained by *m/e* 343. Barring migration of a TMSi group to the pyranose oxygen, or a TMSiO group to C-5, it follows that this ion arises by migration of $(TMSiO)_2PO_2$– to carbon atoms **3** and **4**, probably with retention of the TMSiO– carried by the carbon. The proposed structure of *m/e* 343 is supported by the shift of this ion to

Table II. Fragment Ion Mass Shifts of Deuterium-Labeled TMSi-α-D-Glucose 6-Phosphates[a]

Compound[b]	m/e 103	129	133	147	191	204	211	217	227
TMSi-α-D-Glucose-	—	—	—	—	—	—	—	—	—
6-P (**1**)	10	42	12	53	17	100	10	16	8
TMSi-α-D-Glucose-	0, 1	1, 2	0	0	0	0	0	0	0
6-P-6,6-d_2 (**2**)	10, 0.3	4.4, 38	13	51	17	100	8.5	16	6
TMSi-α-D-Glucose-	1, 2	3, 4	0	0	0, 1	1, 2	0	0, 1, 2, 3	1
6-P-3,4,5,6,6-d_5 (**3**)	6, 4	8, 35	35[c]	44	6, 14	74, 26[d]	8	1, 2, 14, 6	6
TMSi-α-D-Glucose-	1, 2	3, 4	0	0	1	2	0	3	1
6-P-1,2,3,4,5,6,6-d_7 (**4**)	6, 6	8.9, 42	42[c]	49	19	100	8.5	17	6.5
TMSi-d_9-α-D-Glucose-	9	6, 9	9	15	18	18	12	18	15
6-P (**5**)	4	5.9, 43	8	35	15	100	5.2	16	5.2

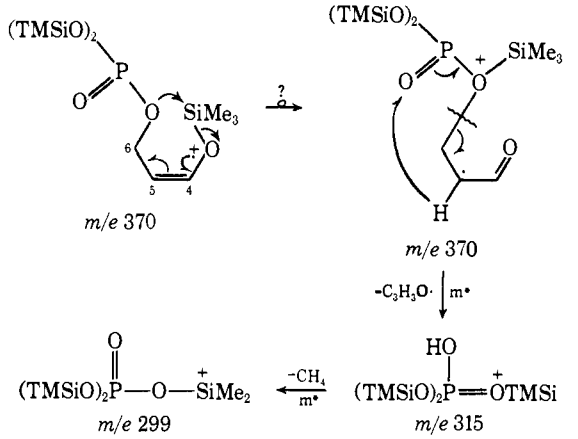
[a] Entries in the table: upper entry is the increase in mass of deuterium-labeled species relative to the unlabeled ion; lower entry is the abundance, in per cent, relative to the base peak. [b] TMSi signifies a completely trimethylsilylated derivative (ether and ester); P signifies phosphate; TMSi-d_9 signifies the derivative completely silylated with trimethylsilyl-d_9 groups. [c] Mixed peak consisting of the d_4 species of

m/e 370 (6.2% abundance relative to *m/e* 342, the TMSi-d_9 counterpart of *m/e* 315) in TMSi-d_9 labeled fructose 6-phosphate. The same shift is observed in TMSi-d_9 labeled ribose 5-phosphate.



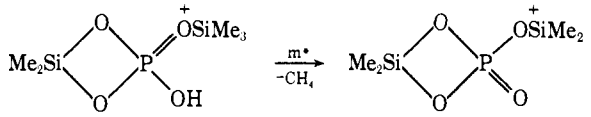
m/e 343

McCloskey and coworkers[10] have reported ions at *m/e* 299 and 315 in the mass spectrum of 5′-adenosine monophosphate. We have observed these ions in the spectra of all of the TMSi sugar phosphates which we have examined. The spectra of TMSi hexose and pentose phosphates contain metastable ions which relate *m/e* 299 to *m/e* 315 [m* 284.0 (calcd 283.9)] and *m/e* 315 to *m/e* 370 [m* 268.5 (calcd 268.2)]. The spectra of the TMSi inositol phosphates,[1] lacking the terminal methylene of hexoses and pentoses, lack *m/e* 370; however, 315 and 299 are present as is the 284.0 metastable ion. The data in Table II support the structures we have given these ions and suggest that *m/e* 370 is derived from C-4, -5, and -6 of glucose 6-phosphate. The spectrum of **3** (Table II) also contains this ion, shifted to *m/e* 374. A possible pathway which could produce these ions is given below. The β elimination of *m/e* 315 from *m/e* 370 is shown as a typical McLafferty rearrangement such as is thought to occur in the mass spectrum of diethyl *n*-pentylphosphonate.[11]



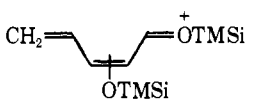
The ion *m/e* 314 appears in all the spectra studied in this paper as well as in the spectra of TMSi inositol phosphates.[1] This ion is shifted by 27 mass units in the spectrum of **5** and appears to be identical with the molecular ion in the spectrum of tris(TMSi)phosphate.[7] In the TMSi inositol phosphates a metastable ion (calculated and found at *m/e* 161.2) relates *m/e* 314 to *m/e* 225, a transformation involving the loss of TMSiO· from *m/e* 314.

Two other phosphorus-containing ions which are related by a metastable peak are *m/e* 227 and *m/e* 211 [(m* 196.1 (calcd 196.1)]. The mass spectrum of **4** contains an ion at *m/e* 228 which corresponds to *m/e* 227 in unlabeled **1** (Table II). This shift is not observed in the mass spectrum of **2** but is formed by the β elimination of bis(TMSi)phosphate from **1** in a McLafferty rearrangement, accompanied by the loss of CH_3·. Both *m/e* 227 and 211 are observed in the mass spectrum of tris(TMSi)phosphate.[7] Structures which accommodate the empirical formulas of these ions are given below. It should be noted that it is possible to draw a symmetrical structure for *m/e* 211; however, because of its mode of formation, the first product ion would most likely be that shown.



m/e 227 *m/e* 211

Many of the remaining ions in the mass spectrum of **1** are found in the spectrum of TMSi-D-glucose.[6] Still others are ions common to TMSi carbohydrates and to other TMSi-derivatives.[5,6,8,9,12,13] Among the ions seen in both TMSi-glucose and **1** is *m/e* 243. The deuterium labeling in Table II suggests that this ion has lost C-1 of **1**.



m/e 243

The major peak in the mass spectrum of **1** (second in abundance to *m/e* 73) is *m/e* 204. This ion is a major fragment in the spectra of many TMSi carbohydrates. The deuterium-labeling studies reported in Table II

(11) J. L. Occolowitz and J. M. Swan, *Aust. J. Chem.*, **19**, 1187 (1966); T. Nishikawa, *Tetrahedron*, **22**, 1383 (1966).

(12) O. S. Chizov, N. V. Nolodstov, and K. N. Kochetkov, *Carbohyd. Res.*, **4**, 273 (1967).

(13) J. Diekman, J. B. Thomson, and C. Djerassi, *J. Org. Chem.*, **33**, 2271 (1968).

m/e													
243	255	270	271	299	315	345	357	370	387	497	512	587	677
—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	1	5	12	46	31	6	25	15	76	1	1	1	2
2	2	2	2	0	0	2	2	2	0	2	2	2	2
6	1	6	13	42	24	5	21	14	76	0.5	0.4	1	2
4	—	—	—	0	1	3, 4	3	4	0	—	—	4, 5	5
3	—	—	—	40	20	1, 3	26	14	85	—	—	2, 0.2	3
5	4	4	5	0	1	5	3	4	0	5	5	6	7
4	0.5	3	12	43	22	5	27	13	81	0.4	t	2	2
18	15	18	18	24	27	24	27	27	36	—	—	42	—
6	1	5.2	10	41	21	4	19	14	72	—	—	1	–

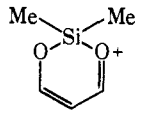
m/e 129 and unlabeled *m/e* 133. [d] Base peak is taken as the sum of *m/e* 204 and 205. The abundance of *m/e* 205 relative to *m/e* 204 is corrected for naturally occurring isotopic contributions.

confirm the structure of *m/e* 204 to be [TMSiOCH=CHOTMSi]$^{\cdot+}$, as reported by others.[6,12,14,15] We observe, as have Curtius, *et al.*,[16] and DeJongh, *et al.*,[6] that *m/e* 204 is characteristically of reduced intensity in carbohydrates which have the furanoside structure. We find this ion to be a major component of the spectrum of TMSi-α-D-mannopyranose 6-phosphate (base peak *m/e* 204) and TMSi-α-D-galactopyranose 6-phosphate (Table III) as well as the spectrum of **1**. In contrast the spectra of TMSi-β-D-fructose 6-phosphate (Table I) and TMSi-β-D-galactofuranose 6-phosphate (Table III) have lesser amounts of *m/e* 204. The two-carbon fragment *m/e* 204 is thus a generally useful predictor of the pyranose ring. We have observed *m/e* 204 to be the ion second to *m/e* 73 in abundance in the major gas chromatographic peaks of the following TMSi sugars, each of which was prepared from the crystalline carbohydrates (non-equilibrated): D-mannose, D-galactose, D-tagatose, D-fructose, D-fucose, L-rhamnose, D-ribose, D-lyxose, and D-xylose. Thus each of these TMSi ethers appears to be predominantly in the pyranose form.

As did DeJongh, *et al.*,[6] we observe a metastable ion for the transformation *m/e* 204 $\xrightarrow{*}$ *m/e* 189 + $CH_3\cdot$ [m* 175.2 (calcd 175.1)]. DeJongh, *et al.*, report that *m/e* 204 originates with C-2–C-3 and C-3–C-4 of TMSi-glucose. Table II shows C-6 not to contribute to 204 in the spectrum of **1**. Of greater interest is the spectrum of **3** which shows *m/e* 204 shifted to *m/e* 205 and *m/e* 206. From this it appears that, in the spectrum of **1**, *m/e* 204 derives to the extent of 74% from C-3–C-4 and 26% from C-2–C-3.

The ion *m/e* 129 has also been observed by DeJongh, *et al.*,[6] in the spectrum of TMSi-D-glucose. This ion was shown to be a doublet with two-thirds of the peak accounted for by a fragment having the structure CH_2=CHCH=OTMSi$^+$ and one-third by an ion of composition $C_5H_9O_2Si^+$. Table II shows that both of these ions appear in the spectrum of **1** with $C_5H_9O_2Si^+$ accounting for one-tenth of the mixed peak. A possible structure for this ion is shown.

An ion at *m/e* 133 is found in the spectrum of **1** and of all the other TMSi carbohydrate phosphates we have studied. This ion is also observed in the mass spectrum of tris(TMSi)phosphate[7] where it clearly has the composition $(CH_3)_3Si_2O_2^+$. Table II bears out this ion composition in the spectrum of **1** also.



m/e 129

The balance of the ions in the mass spectrum of **1** is commonly seen in the spectra of TMSi alcohols.[5,6,8,10,15,16] These include *m/e* 73 (Me_3Si^+), 75 ($HOSiMe_2^+$), 103 (Me_3SiO=CH_2^+), 147 (Me_3SiO-$SiMe_2^+$), 191 (Me_3SiOCH=$OSiMe_3^+$), 217 (Me_3SiO-CH=CHCH=$OSiMe_3^+$), and 305 (Me_3SiOCH=C-($OSiMe_3$)CH=$OSiMe_3^+$). Table II is in agreement with other workers as to the structures of *m/e* 103, 147, 191, and 217. Similar data, not included in Table II, support the structures of *m/e* 73, 75, and 305. It is of interest that Table II shows the label from **2** not to be incorporated into *m/e* 191 and 217. DeJongh, *et al.*,[6] have shown *m/e* 191 to retain C-1 of TMSi-glucose to the extent of about 90%. We have observed in the spectrum of **4** that one deuterium atom is retained by *m/e* 191 and that TMSi-D-glucose 6-phosphate-3,4,5,6,6-d_5 introduces no label into this ion. It is also of interest to note that **2** contributes little, if any, deuterium to *m/e* 103, clearly making this a rearrangement ion also.

We were interested in determining if α- and β-anomers of **1** were mass spectrally distinguishable and if the spectrum of **1** differed significantly from isomers such as TMSi-D-mannose 6-phosphate. To a degree, both of these characterizations can be made. The precision of our measurement of ion abundances is ±10% for peaks of 5% or greater relative intensity. Several ions have differences in abundance, for α and β forms of the same sugar, and for the same form of two isomeric sugars, which are significant by this criterion. One of these ions is *m/e* 299 which has a relative intensity of 46% for α-**1** and 54% for β-**1**. This ion has an abundance of 60% for TMSi-α-D-mannose 6-phosphate. Several other ions show similar differences which must arise from the stereochemistry of the molecular ion and its effect on the process of fragmentation.

The Mass Spectrum of Penta-O-trimethylsilyl-β-D-ribofuranoside 5-Phosphate (6) and Hexa-O-trimethylsilyl-β-D-fructofuranoside 6-Phosphate (7). The bar graph presentation of the spectrum of **7** is shown in

(14) N. K. Kochetkov, O. S. Chizov, and N. V. Molodtsov, *Tetrahedron*, **24**, 5587 (1968).
(15) G. Petersson and O. Samuelson, *Svensk. Papperstid*, **71**, 77 (1968).
(16) H. C. Curtius, J. A. Vollmin, and M. Muller, *Z. Anal. Chem.*, **243**, 341 (1968).

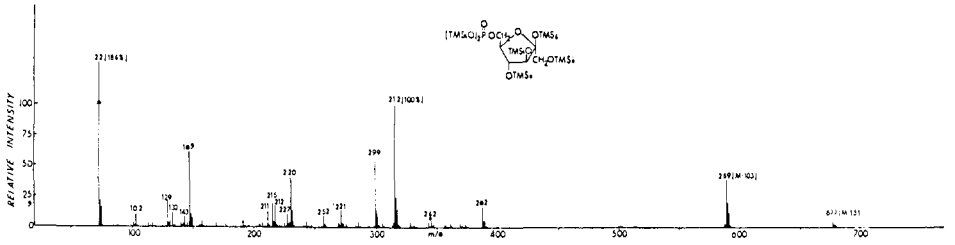


Figure 2. The 70-eV mass spectrum of hexakis(trimethylsilyl)-β-D-fructofuranose 6-phosphate (**7**), mol wt 692.

Figure 2. Once again, the base peak is chosen to be the second most abundant ion, *m/e* 315. The spectrum of **6** is similar to **7** with the same base peak and with minor differences which will be discussed. Tabulation of the mass spectra of these compounds is found in Table I. TMSi-β-D-fructose 6-phosphate-6,6-d_2 and -3,4,5,6,6-d_5 were also examined and are discussed in the text.

The mass spectra of **6** and **7** contain only trace amounts of the molecular ion. Once again, the M − 15 ions are the highest mass fragments of useful intensity. Metastable ions are largely lacking and most structural assignments must be made with the aid of deuterium labeling.

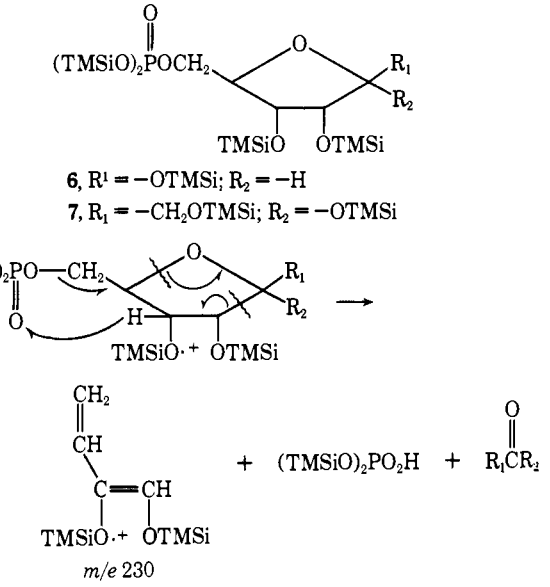
MeCloskey, *et al.*,[10] suggest that *m/e* 230 is characteristic of ribonucleosides. We find this ion in the spectra of both **6** and **7**.[17] It is significant that *m/e* 230 is an ion of relatively minor abundance in the spectrum of both TMSi-α-D-galactofuranose 6-phosphate and TMSi-α-D-galactopyranose 6-phosphate (Table III).

Table III. Fragment Ions of TMSi-D-Galactopyranoside 6-Phosphate and TMSi-D-Galactofuranoside 6-Phosphate[a]

m/e	Intensity relative to *m/e* 73	
	Furanoside	Pyranoside
73	100	100
129	14.8	25.6
169	2.7	1.4
191	11.1	12.6
204	3.6	41.8
215	1.6	0.5
217	61.0	18.3
230	1.6	1.0
315	27.6	13.7
331	2.0	0.6
332	10.2	0.9
343	11.9	0.0
357	2.3	19.5
387	39.2	39.2

[a] When commercial galactose 6-phosphate is trimethylsilylated and gas chromatographed, four peaks of approximately equal size are obtained. Mass spectral analysis of these peaks show the first two to be the furanose and the second pair the pyranose forms of this sugar.

It is also a minor ion in the spectrum of TMSi-fructose 1-phosphate where it has an abundance of 5% relative to *m/e* 73.[18] Furthermore, this ion appears in only small amounts in the spectra of nonphosphorylated TMSi-fructose[16] and TMSi-ribose.[18] From these observations it seems that *m/e* 230 is most readily formed from ketohexofuranoside 6-phosphates and pentofuranoside 5-phosphates, *i.e.*, sugars with the 5 position of the furanose ring bearing the phosphorylated methylene carbon. The structure proposed for *m/e* 230 is supported by the spectra of TMSi-fructose 6-phosphate-6,6-d_2, and TMSi-fructose 6-phosphate-3,4,5,6,-6-d_5. The shift of *m/e* 230 to *m/e* 234 in both cases shows the deuterium atoms of C-6 to be left intact. Labeling of **6** and **7** with TMSi-d_9 groups shows *m/e* 230 to have two TMSi moieties.



6, $R_1 = -OTMSi$; $R_2 = -H$

7, $R_1 = -CH_2OTMSi$; $R_2 = -OTMSi$

m/e 230

An ion which is abundant in the spectrum of **6** and only a minor ion in all other TMSi sugar phosphates discussed in this paper is *m/e* 169, the counterpart of *m/e* 271 in the spectra of **1** and **7**. McCloskey, *et al.*,[10] have observed this ion to be severalfold more intense in the spectrum of TMSi-5′-adenosine monophosphate than it is in the spectrum of TMSi-guanosine. In the spectrum of TMSi-d_9-ribose 5-phosphate this ion is shifted to *m/e* 178 and has an abundance of 23% relative to *m/e* 342 (the TMSi-d_9 counterpart of *m/e* 315). Scheme II shows two possible routes to *m/e* 169. The spectra[18] of TMSi-arabinose, -lyxose, -rhamnose, -ribose, and -xylose have 2% or less of *m/e* 169 strengthening the idea that its enhanced abundance in TMSi sugar phosphates is due to the ease of elimination of neutral bis(TMSi)phosphate. The spectrum of TMSi-d_9-ribose 5-phosphate contains each of these ions shifted in

(17) The spectra of **6** and **7** have a metastable ion at *m/e* 201.0 which could be formed during the loss of methyl radical from *m/e* 230 to form *m/e* 215 (calcd m* 201.0). The ion *m/e* 215 is relatively abundant in **6** and **7** and is shifted to *m/e* 217 (30%) in the spectrum of TMSi-D-fructose 6-phosphate-6,6-d_2. The spectrum of TMSi-D-fructose 6-phosphate-3,4,5,6,6-d_5 appears to have 215 shifted to both *m/e* 217 (20%) and *m/e* 219 (9%). Finally, in the spectra of TMSi-d_9-ribose and -fructose phosphate the expected shift to *m/e* 230 is not observed, nor does any *m/e* 215 peak remain. At maximum resolution of our instrument (1000, 10% valley) *m/e* 215 appears to be a composite peak; thus the various fragments resulting from TMSi-d_9 labeling may be dispersed and lost in other fragment ions.

(18) Unpublished results from this laboratory.

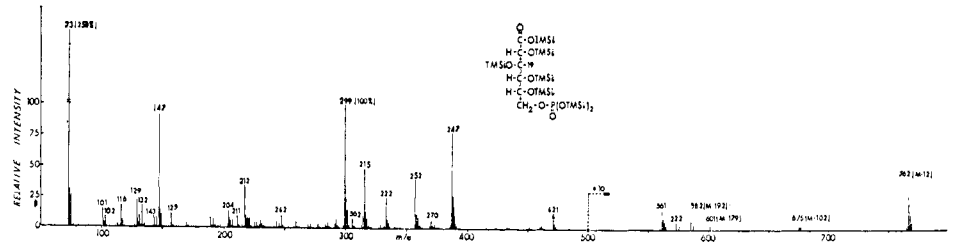
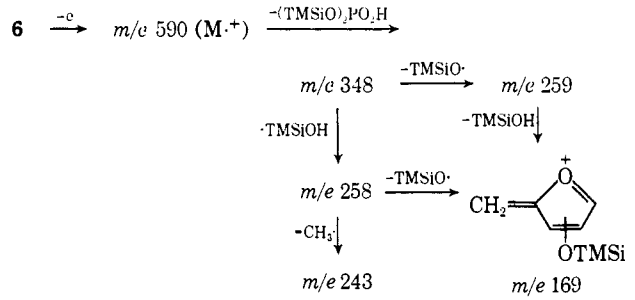


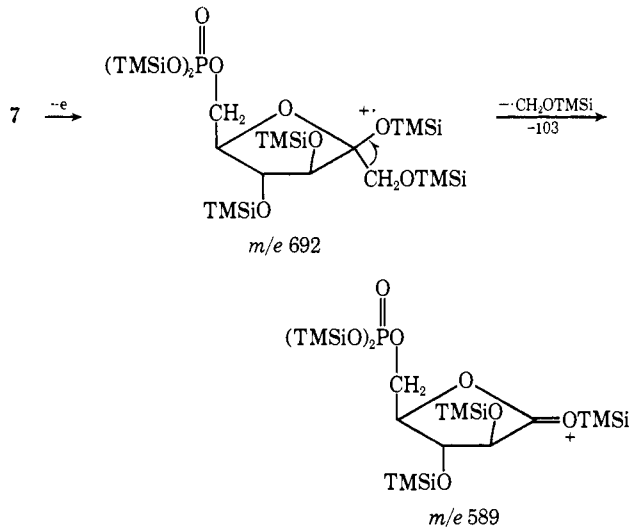
Figure 3. The 70-eV mass spectrum of heptakis(trimethylsilyl)-6-phosphogluconate (**8**), mol wt 590.

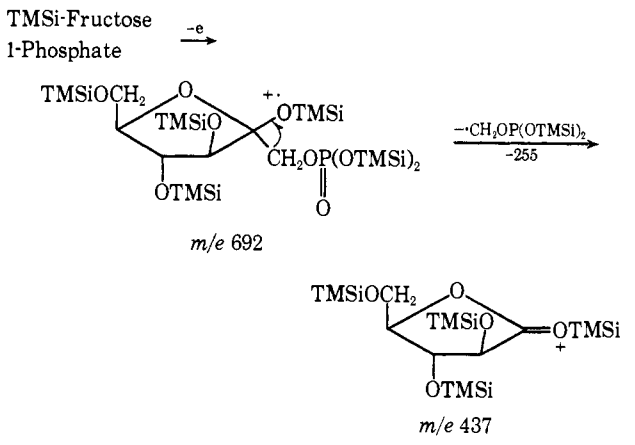
a way which makes their structures compatible with the scheme proposed below. This labeling shows the *m/e* 243 of this sequence to be different from that observed in the spectrum of **1**.

Scheme II



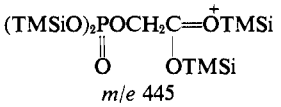
The mass spectrum of **7** has a particularly abundant ion at *m/e* 589 which is seen only in trace amounts in the spectra of **1** and mannose 6-phosphate and in the spectra of TMSi-galactopyranose and -galactofuranose





6-phosphate. The spectrum of TMSi-fructose 6-phosphate-6,6-d_2 contains this ion shifted to *m/e* 591 in 51% relative abundance. In the spectrum of TMSi-fructose 6-phosphate-3,4,5,6,6-d_5 *m/e* 591 is shifted to *m/e* 594 in 45% abundance. Curtius, *et al.*,[16] have observed an ion at *m/e* 437 in the spectrum of TMSi-fructose which is produced by an analagous process, the loss of $\cdot CH_2OTMSi$. A similar fragmentation occurs in the spectrum of TMSi-D-fructose 1-phosphate where $\cdot CH_2OPO(OTMSi)_2$ is lost to produce *m/e* 437 (5.3% relative to *m/e* 73). TMSi-d_9 labeling is consistent with the structures of both *m/e* 589 and *m/e* 437.

Another ion found in very high abundance (75% relative to *m/e* 73) in the spectrum of TMSi-fructose 1-phosphate is *m/e* 445. TMSi-d_9 labeling shows this ion to carry four TMSi groups and suggests that it has a structure analagous to *m/e* 357 in the spectrum of **1**.



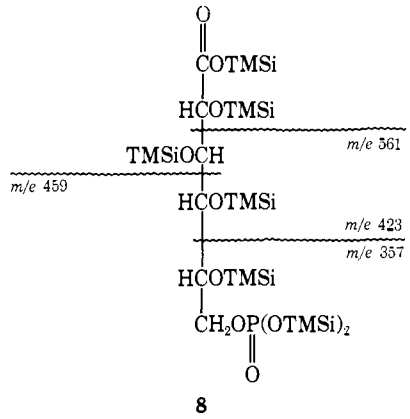
The high abundance of this rearrangement ion in the spectrum of TMSi-fructose 1-phosphate could result from the ease with which the 6-TMSi group may migrate to the furanose ring oxygen.

The base peak chosen in the spectrum of **6** and **7** is at *m/e* 315. The metastable ions which relate the transition of *m/e* 370 to *m/e* 315 and the decomposition of 315 to *m/e* 299 are observed in the spectra of both **6** and **7** as they were in the spectrum of **1**.

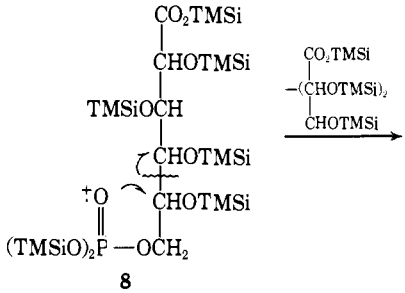
The Mass Spectrum of Hepta-*O*-trimethylsilyl 6-Phosphogluconate (8). The bar graph presentation of the mass spectrum of **8** is given in Figure 3 with *m/e* 299 taken as the base peak. The tabulated spectrum of **8** is in Table I. The highest mass ion of useful abundance in the spectrum of **8** is M − 15 (*m/e* 765). This ion is shifted to *m/e* 825 [found 825.5 (calcd 825.6)] by labeling with TMSi-d_9.

Unlike the mass spectrum of aliphatic carboxylate trimethylsilyl esters[19] **8** does not undergo loss of methyl radical followed by decarboxylation. The main processes which **8** undergoes are loss of TMSiO· and TMSiOH often accompanied by chain cleavage as outlined below. Each of these ions, with the exception of *m/e* 357, undergoes loss of TMSiOH. In the case of *m/e* 561 → *m/e* 471 a metastable ion is observed [395.6 (calcd 395.4)]. The high abundance of *m/e* 357 may be the result of a facilitated cleavage of the C-5–C-6 bond as shown below.

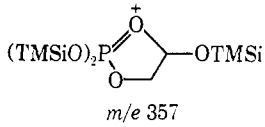
(19) R. M. Teeter, Abstracts, Tenth Annual Conference on Mass Spectrometry and Allied Topics, New Orleans, La., June 1962.



8



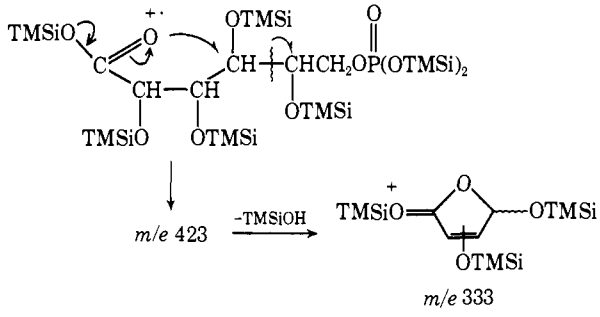
8



m/e 357

The structures of each of the ions in this series are supported by the mass shifts observed following TMSi-d_9 labeling.

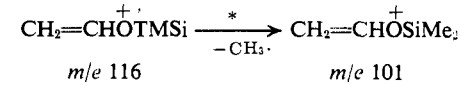
Two ions, *m/e* 423 and *m/e* 333, in the series do not contain phosphorus. The high abundance of *m/e* 333 in the spectrum of **8** relative to the spectra of **1**, **6**, and **7** may be the result of the following process which is unique to **8**.



m/e 333

Two more metastable ions are present in the spectrum of **8**, one for the fragmentation of *m/e* 459 → 387 [m* 326.5 (calcd 326.3)], the other for the process *m/e* 315 → 299 [m* found 284.0 (calcd 283.9)]. These ions have been discussed in the section devoted to the mass spectrum of **1**.

Two other ions which appear in the spectrum of **8** but which are relatively minor fragments in the spectra of **1**, **6**, and **7** are *m/e* 116 and *m/e* 101. The ion *m/e* 101 is formed from *m/e* 116 by the loss of a methyl radical for which a metastable ion at *m/e* 88.0 is observed (calcd 87.9). It is probable that these ions are formed from C-6 and C-5 of **8**. Labeling with TMSi-d_9 shifts *m/e* 116 to *m/e* 125 and *m/e* 101 to *m/e* 107, in agreement with the structures proposed. These ions are also seen in the spectrum of TMSi-sorbitol 6-phosphate in an abundance of 11% (*m/e* 101)



m/e 116 *m/e* 101

and 10% (*m/e* 116).[18] It is interesting that they also appear, to a small degree, in the spectra of TMSi-glucose and -mannose 6-phosphate both of which are known to be exclusively in the pyranose form (by gas chromatographic separation). These sugars, which are 1,5 acetals, are unable to give rise directly to *m/e* 116 ions and must, therefore, undergo trimethylsilyl migration. A similar event must occur in the spectra of **6** and **7**. The spectra of TMSi-sorbitol 6-phosphate-6,6-d_2 and -1,2,3,4,5,6,6-d_7 also support the structures proposed for *m/e* 116 and 101.[18]

Summary of the Features of Biochemical Interest Present in the Mass Spectra of TMSi-Sugar Phosphates. One of the aspects of mass spectra useful to biochemists is the ability to identify many fragment ions with respect to their specific origin in the molecule, providing a route for the identification of specific isotope labels. Stable isotope labeling studies many times have advantages over radioactive tracer labeling. An example is the simpler evaluation of the kinetics of certain enzymatic processes by the identification and measurement of primary isotope rate effects, *e.g.*, during the breaking of a C–H *vs.* a C–D bond (*e.g.*, ref 24). Listed below are the ions found in the mass spectra of the TMSi-sugar phosphates which we have studied and which have been identified with respect to their origins in the parent sugars. Each of these ions retains all of the hydrogen atoms borne by the original carbon atoms of the intact sugar except as indicated. The M − 15 ion in the spectrum of each sugar phosphate (Table I) carries all of the original sugar C–H groups.

TMSi-Glucose 6-Phosphate. C-2, -3, -4, -5, and -6 including 2 CH, 6 CH_2, and two hydrogens from the remaining carbon atoms are carried by *m/e* 243; C-4, -5, and -6, by *m/e* 370; C-2–C-3 (74%) and C-3–C-4 (26%) by *m/e* 204; C-5–C-6 by *m/e* 357; and C-3 or C-4 by *m/e* 343.

TMSi-Fructose 6-Phosphate. C-2, -3, -4, -5, and -6 including 2 CH, 6 CH_2, and two hydrogens from the remaining carbon atoms are carried by *m/e* 243; C-2, -3, -4, -5, and -6 by *m/e* 589; C-3, -4, -5, and -6 including 6 CH_2 and two hydrogens from the remaining carbon atoms by *m/e* 230; C-5–C-6 by *m/e* 357; and C-3 or C-4 by *m/e* 343.

TMSi-Fructose 1-Phosphate. No carbon chain labeling was performed; however, the fragmentation similarities with TMSi-fructose 6-phosphate strongly suggest that C-2, -3, -4, -5, and -6 are carried by *m/e* 437. The abundant ion *m/e* 445 probably carries C-1–C-2, thus only the C-1 hydrogens of fructose 1-phosphate.

TMSi-Ribose 5-Phosphate. No carbon chain labeling studies were made; however, in analogy with the other spectra C-3, -4, and -5 including 5-CH_2 and two hydrogens from the remaining carbon atoms are present in *m/e* 230; C-4–C-5 in *m/e* 357; and C-3 or C-4 in *m/e* 343.

TMSi-6-Phosphogluconate. Again, no chain labeling was performed; however, the fragmentation pattern suggests that C-3, -4, -5, and -6 are carried by *m/e* 561;

C-1, -2, -3, and -4 by *m/e* 423; C-4, -5, and -6 by *m/e* 459; and C-5–C-6 by *m/e* 101 and *m/e* 116.

As Curtius, *et al.*,[16] and DeJongh, *et al.*,[6] have shown, certain ions are useful indicators of the presence of the pyranoside and furanoside forms of TMSi carbohydrates. We observe this to be true of the TMSi phosphorylated sugars also. The most reliable of these ions is *m/e* 204, which in all cases thus far studied, is of low intensity in the furanoside sugars and high intensity when the pyranoside form is present.

m/e 169 is an ion which is found in high abundance in the spectrum of TMSi-ribose 5-phosphate. While no other pentose phosphates have been studied, the absence or very low abundance of this ion in the spectra of all other sugars we have studied suggests strongly that it is unique to the pentose phosphates.

We and McCloskey, *et al.*,[10] have found *m/e* 230 to be of high abundance in the spectra of compounds where TMSi-ribose 5-phosphate is present. This ion is also present in high abundance in the spectra of TMSi-fructose 6-phosphate but not in TMSi-fructose 1-phosphate.

From its structure and mode of formation, *m/e* 589 appears to be most readily formed from TMSi-2-ketohexofuranose 6-phosphates. Similarly, *m/e* 437 seems likely to be particularly abundant in only the TMSi-2-ketohexofuranose 1-phosphates.

One of the techniques which combined gas chromatography–mass spectrometry has made possible, and which has considerable importance to biochemists, is the use of the combined instrument as a selective ion detector for the gas chromatograph.[20] The extreme sensitivity of the combined instrument when used with an abundant ion[21] often makes this the method of choice for the detection, identification, and measurement of trace amount substances. Although most fragment ions are only unique in a relative sense, careful selection with consideration of other factors can increase the selectivity while maintaining a high degree of sensitivity. The six types of phosphorylated sugars we have studied may be usefully detected by the following relatively unique ions (Tables I and III contain many other ions of similar usefulness): TMSi-aldohexopyranose 6-phosphates, *m/e* 204; TMSi-aldohexofuranose 6-phosphates, *m/e* 332 and 343; TMSi-2-ketohexofuranose 1-phosphates, *m/e* 445; TMSi-aldopentose 5-phosphates, *m/e* 169; TMSi-hexonate 6-phosphates, *m/e* 333.

In instances where a study of the hydrogen–deuterium ratio of a TMSi sugar is to be made the M − 15 ion is generally of greatest use. This ion is usually in a region free of gas chromatograph column bleed and generally has no other ions of consequence at nearby *m/e* values. Since the naturally abundant heavy isotopes of C, H, O, and Si make considerable contribution to the spectra of TMSi sugars, it is often necessary to determine the contribution of these ions empirically or by calculation,[22] in order to evaluate the results of deuterium labeling.

Experimental Section

D-Glucose 6-phosphate, D-mannose 6-phosphate, D-galactose 6-phosphate, D-fructose 6-phosphate, D-fructose 1-phosphate, D-ribose 5-phosphate, and 6-phosphogluconic acid were purchased from the Sigma Chemical Co., St. Louis, Mo., as sodium or cyclohexylamine salts and used without further purification. D-Glucose-6,6-d_2 and D-glucose-1,2,3,4,5,6,6-d_7 were purchased from Merck Sharp and Dohme of Canada, Ltd., Montreal, and phosphorylated at the 6 position using hexokinase and adenosine triphosphate obtained from the Sigma Chemical Co. The enzymatic procedure followed that of Melo, *et al.*[23] D-Glucose 6-phosphate-3,4,5,6,6-d_5, D-fructose 6-phosphate-3,4,5,6,6-d_5, and D-fructose 6-phosphate-6,6-d_2 were prepared by the action of an enzyme preparation from rat or beef testis[24] which contained phosphoglucoisomerase and phosphomannoisomerase that catalyze the reaction D-glucose 6-phosphate ⇌ D-fructose 6-phosphate ⇌ D-mannose 6-phosphate. During the course of this equilibrium reaction deuterium labels at C-1–C-2 were lost to the aqueous reaction medium. Sorbitol 6-phosphate-6,6-d_2 and -1,2,3,4,5,6,6-d_7 were obtained by sodium borohydride reduction of the deuterated glucose phosphates.

Deuterium labeling of various TMSi-carbohydrates as their poly-TMSi-d_9 derivatives was carried out using d_{18}-bis(trimethylsilyl)acetamide prepared and used according to McCloskey, *et al.*[10] This reagent is now commercially available from Merck Sharpe and Dohme of Canada, Ltd., Montreal.

Samples were prepared for gas chromatography by treatment of 2 μmoles of the sugar phosphate (as the sodium or cyclohexylammonium salt) with 50 μl of bis(trimethylsilyl)acetamide, 20 μl of trimethylchlorosilane, and 30 μl of pyridine which had been dried over Molecular Sieves 5A (Linde). This is essentially the method used by McCloskey, *et al.*[10] After about 1 hr at room temperature aliquots containing 0.01 μmole were injected onto the gas chromatographic column of an LKB 9000 gas chromatograph–mass spectrometer (LKB Produkter, Stockholm-Bromma, Sweden). The gas chromatography was carried out on a 4 ft × 0.25 in. glass column packed with etched glass beads (80–100 mesh, code 0201, Corning Glass Works, Corning, N. Y.) coated with 0.1% w/w Dow Corning DC-710 silicone oil. We have subsequently found that OV-17 on silanized diatomaceous earth supports is similar in retention characteristics and more reliable than DC-710 on glass beads. The chromatography was at 170° with a helium carrier flow of 30 cc/min.

When these studies began, we were unable to achieve transfer of the eluate from the gas chromatograph column to the ionizing source of the spectrometer. We then replaced the standard porous ceramic insulating duct between the source and the molecule separator of the spectrometer with a siliconized glass duct, which we prepared. Following this, samples were generally transferred satisfactorily; however, difficulties are still encountered with TMSi carbohydrate diphosphates. These losses are reminiscent of those experienced by Quayle[25] in the mass spectrometry of organic phosphate esters. Quayle believed that this was the result of adsorption of the phosphorylated compound with degradation to a substance (phosphoric acid?) which catalyzed the decomposition of samples introduced subsequently.

Spectra were all obtained at 70 eV ionizing potential, a trap current of 60 μA, an accelerating voltage of 3.0 or 3.5 kV, and slits of 0.08 and 0.20 mm. The source was maintained at 270°. Scans were taken at an average rate of 77 amu/sec with 120-cps filtering. *m/e* values were assigned by reference to perfluorokerosene introduced into the source during or following the elution of the sample. In later work, an LKB mass marker was used, which was accurate up to and beyond the highest mass measured (*m/e* 825). The accelerating voltage was 3.5 kV when using the mass marker.

Metastable peaks were located using five to ten times larger samples with 3.5 kV accelerating potential and slits of 0.1 and 0.3 mm.

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The Inactivation of α-Chymotrypsin with Methyl-, Trideuteriomethyl-, and Trifluoromethyl-Substituted N-Phenyl-α-bromoacetamides

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Abstract: The kinetics of the irreversible reaction of *o*-, *m*-, and *p*-methyl- and -trifluoromethyl-substituted N-phenyl-α-bromoacetamides with α-chymotrypsin have been studied at pH 6.0. Dissociation constants for the initially formed enzyme–inhibitor complexes have been determined or estimated and rate constants for reaction of these complexes to give inhibited proteins have been obtained. The *para* position seems to be most sensitive to substitution; a methyl group at this position enhances the stability of the enzyme–inhibitor complex but does not affect the rate of subsequent reaction while a *p*-trifluoromethyl group at this position greatly reduces both binding and this reaction rate. The behavior of the *p*-trideuteriomethyl-substituted inhibitor is indistinguishable from that of the protio compound.

An important adjunct to the traditional use of reaction kinetics for the study of enzymic active sites is the chemical modification of amino acids in the enzyme. With the ability to alter specific amino acid residues, often near the active center of the protein, one has a potentially powerful tool for the study of local environments in the enzyme, especially if the modification affords a protein with physical properties amenable to study by spectroscopic techniques. The use of "reporter groups" for the exploration of the active site of α-chymotrypsin is rather well developed.[2-6] Although some use has been made of proton magnetic resonance spectroscopy to investigate this enzyme,[7-9] one cannot expect a great deal of success in the reporter group type of study by this technique since the proton signals of the reporter group will be obscured by the large number of proton resonances of the enzyme itself. We have, therefore, embarked upon the preparation of modified α-chymotrypsins which contain fluorine or deuterium in the reporter group, with the hope that nmr studies of these labeled materials will provide new information about the properties of this enzyme.

Methionine-192 has been a favorite target for investigations which utilize the reporter group idea because of its high reactivity toward "active" halogens. Lawson and Schramm have investigated the irreversible inhibition of α-chymotrypsin by a series of N-substituted α-bromoacetamides as well as α-bromoacetophenone and benzyl bromide.[10] The results of these workers, and others,[2,3,11,12] show that organic molecules with an activated halogen (usually bromine) and a large nonpolar group (often aromatic) will inactivate this enzyme irreversibly by first forming a complex with the active site followed by displacement of the halogen by the sulfur atom of methionine-192. Although the compounds investigated to date inactivate the enzyme at widely different rates, it is usually the methionine-192 residue which is attacked. It has been suggested that this residue is near a "hydrophobic" binding locus;[13] a properly constituted inhibitor molecule may occupy this site *via* a hydrophobic interaction which places the α-bromoacetyl moiety of this species into the correct orientation for reaction with the thio ether linkage of the methionine.

With these observations in mind we prepared a series of methyl and trifluoromethyl substituted N-phenyl-α-bromoacetamides (Ia–h) and have studied their ability to irreversibly inhibit α-chymotrypsin.

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