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Permethylation for Mass Spectrometry: Rearrangements of Ester Linkages and Use of Potassium *t*-Butoxide

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Abstract—Thirteen selected compounds were permethylated with potassium *t*-butoxide in dimethyl sulphoxide and methyl iodide, a simpler procedure than those using sodium hydride but equally effective. From mass spectrometric study of the products, it emerged that carboxylic esters of methanol and of *o*-diphenols were at least partially rearranged, whereas those of benzyl alcohol and of a monophenol, as well as fully acylated carbohydrate-like substances, were largely not. Some anomalies of *C*-methylation and *N*-methylation were encountered.

Introduction

VOLATILITY is the limiting property of all samples submitted for mass spectrometry, except perhaps with the latest field desorption techniques. Volatile derivatives can be prepared which render many involatile substances of biological interest amenable to mass spectrometry and combined gas chromatography-mass spectrometry. Derivatization methods, especially with isotopic labelling reagents, can also yield information about the number and nature of 'functional' groups present. The blocking of these functional groups increases chemical stability and prevents artefactual intermolecular reactions at the rather high temperatures which have to be used. Direct permethylation can be used in such classes as the carbohydrates or flavonoid glycosides,[1,2] but a preliminary acylation is necessary with compounds having basic functions, to prevent formation of nonvolatile quaternary ammonium salts. Acetylation has been used as a preliminary to methylation in the highly successful peptide sequencing studies initiated by Lederer and colleagues; in nearly all this work *N*-acetylation has been performed under conditions which avoid *O*-acetylation of hydroxyl groups, etc. However, in particular cases, preliminary *O*-acetylation may turn out to be useful.

For mass spectrometric study of polyphenolic compounds, initial *O*-acetylation is often the preferred approach[3] and with quinones, useful stabilization may be achieved by reductive acetylation.[4-6]

As an approach to the study of quinone–amine coupling reactions (for a review on biological significance see Lit.[7]) we planned to stabilize reaction products by acetylation and then in suitable cases to permethylate these acetyl derivatives, with a view to enhancing volatility for g.c. and m.s. work, to clarifying the m.s. fragmentation pattern at amide bonds etc. and to manifesting the presence of various functional groups.

Rearrangement by migration of carboxylic *O*-acyl groups, on permethylation of carbohydrate derivatives, has long been known, so it was not surprising to find that methyl esters of pyrrolidonecarboxylic acid and of methylated ascorbalamic acid were rearranged during perdeuteriomethylation to CD_3 esters.[8] As many of the natural products in which we are interested (e.g. chlorogenic acid) themselves contain ester linkages, such rearrangements could cause loss of structural information. We therefore decided to look at the behaviour of ester linkages of varied types, with a view to assessing the range of applicability of this approach.

In most of the more recent studies involving permethylation of peptides, sodium hydride (dissolved in dimethyl sulphoxide, *N*,*N*-dimethylformamide or *N*,*N*-dimethylacetamide) has been used. Dr W. M. Horspool and Professor Lord Tedder suggested to us that potassium *t*-butoxide might prove a useful reagent. It has not so far been much used for *N*- or *O*-alkylations.[9] It is more readily and more safely[10,11] brought into solution and, as shown below, effects methylation at most of the expected sites.

Experimental

The substances studied are listed in Table 1 with structural formulae. Compounds **7** and **11** were commercial preparations. Of those prepared by published methods (references in Table 1), **1** was a gift from Dr E. Haslam and **10** from Mr E. I. Mbadiwe, who also prepared **13** by acetylation of caffeoylputrescine in acetic anhydride–pyridine with subsequent water treatment.*

L-3-*Chloroalanine benzyl ester hydrochloride* (**15**)

L-Serine benzyl ester benzenesulphonate, a gift from Professor G. Fölsch, was converted to the hydrochloride (**14**).[12] This (2.08 g) in freshly distilled acetyl chloride (20 ml) was treated at +4 °C with phosphorus pentachloride (2.16 g) added in three portions with vigorous shaking, which was continued for 30 min at room temperature. The resulting solid (**15**) was filtered

* E. I. Mbadiwe, unpublished work.

off, washed with a little acetyl chloride and then with light petroleum (yield, 1.61 g). M.p. (uncorr.) 134 to 5 °C (decomp.), $[\alpha]_D^{25.8°C} - 41.3°$ (*C*, 3.3 in water). (Found (Pascher, Bonn): C, 47.9; H, 5.32; Cl, 27.6; N, 5.44; O, 13.1. $C_{10}H_{12}ClNO_2$, HCl requires C, 48.0; H, 5.24; Cl, 28.3; N, 5.60; O, 12.8%). Szekerke[13] prepared the racemic compound, using chloroform as the reaction medium. In this work dissolution in chloroform was poor; acetyl chloride[14] proved a better medium.

*N-Benzyloxycarbonyl-3-chloro-*L*-alanine benzyl ester* (**6**)

15 (1.26 g) was carbobenzoxylated in water–chloroform[15] to give crude product (1.22 g). Recrystallized from ethanol, **6** had m.p. (uncorr.) 90 °C, $[\alpha]_D^{27°C} + 13.5°$ (*C*, 3.3 in chloroform). (Found (Pascher, Bonn): C, 62.4; H, 5.24; Cl, 10.1; N, 3.89; O, 18.2. $C_{18}H_{18}ClNO_4$ requires C, 62.2; H, 5.22; Cl, 10.2; N, 4.03; O, 18.4%).

*N^1-Acetyl-*DL*-5-(p-acetoxyphenyl)hydantoin* (**9**)

8 (127 mg) was dissolved in A.R. pyridine (2 ml) and acetic anhydride (2 ml), and kept overnight at room temperature. Excess water was added and the mixture repeatedly evaporated with water *in vacuo* below 40 °C until pyridine could no longer be smelt. The yield was 188 mg of crystals which, after recrystallization from aqueous ethanol, had m.p. (uncorr.) 210 to 2 °C. (Found (Pascher, Bonn): C, 56.21; H, 4.44; N, 10.11; O, 29.28. $C_{13}H_{12}N_2O_5$ requires C, 56.6; H, 4.38; N, 10.16; O, 29.0%). ^{1}H n.m.r. (100 MHz in deuteriodimethyl sulphoxide) showed: τ (relative to TMS): 7.80 (*S*, 3H); 7.60 (*S*, 3H); 4.55 (*S*, 1H); 2.91 and 2.66 (ABq, $J_{AB} = 8.5$ Hz, 4H).

Our evidence for the (expected) acetylation at N − 1 rather than N − 3 is that the product of permethylation (see Tables 1 and 2), on alkaline hydrolysis, gave on ion-exchange chromatography a peak in the position of 2-(*p*-hydroxyphenyl)glycine,[16] indicating that methylation had occurred at N-3 rather than at N-1 or C-5 (see also Discussion).

Permethylation

Up to 5 mg of the material to be methylated was dissolved in 3 drops of dry dimethyl sulphoxide. To this was added 1 ml of a 5.6% (w/v) solution of potassium *t*-butoxide (Phase Separations Ltd, Queensferry, Flintshire) in the same solvent (prepared by heating to 70 °C) and then methyl iodide (0.1 ml). The mixture was kept for 1 h at room temperature. 1 M Acetic acid (1 ml) was then added and the mixture extracted with chloroform (10 ml). The chloroform phase was washed 4 times with equal volumes of water and then evaporated to dryness *in vacuo*. The residues were transferred for m.s. in methanol or methylene dichloride. No further attempt was made to fractionate the reaction products, in the hope that the m.s. would reveal minor as well as major reactions.

Mass spectrometry

Low resolution mass spectra were obtained on a MS-902 mass spectrometer (A. E. I. Ltd, Manchester), using the direct insertion probe, a source temperature of 200 °C and an ionization energy of 70 eV.

Results

The reactions studied are set out in Table 1, with summarized interpretations and with references to relevant literature. The complete mass spectra of compounds **1** to **15**, where novel, have been deposited at the Mass Spectral Data Centre, Aldermaston, Berkshire, England. Summarized mass spectra of some of the initial compounds and permethylation products are in Table 2. Several of the latter indicate the presence of more than one compound. The detailed spectra of

TABLE 1. Mass spectrometric study of permethylation reactions

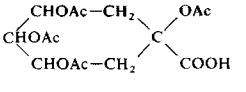
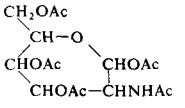
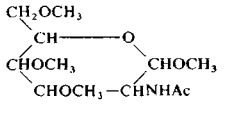
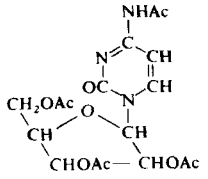
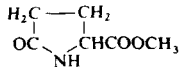
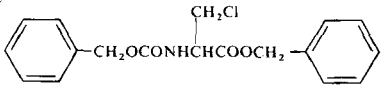
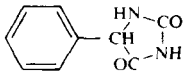
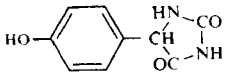
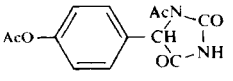
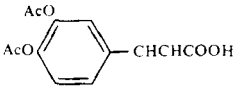
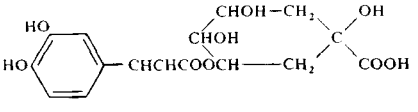
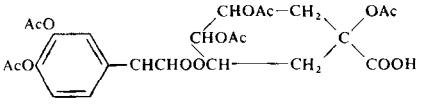
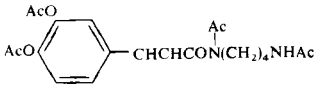
No.	Compound	Formula	Lit. refs. Preparation	Lit. refs. M.s.	Permethylation products: Δ Ac groups	Permethylation products: Δ Me groups	Permethylation products: Interpretation	Permethylation products: Lit. refs. Preparation	Permethylation products: Lit. refs. M.s.
1	1,3,4,5-Tetra-*O*-acetyl-D-quinic acid		25	cf. 26	0	+1	Conversion to Me ester	—	—
2	*N*-Acetyl-1,3,4,6-tetra-*O*-acetyl-2-amino-2-desoxy-β-D-glucopyranose		27	28, cf. 45	0	0	No reaction	27	28
3	Methyl *N*-acetyl-3,4,6-tri-*O*-methyl-2-amino-2-desoxy-β-D-glucoside		29	30,31	0	+1	*N*-Me derivative formed	32,33,41	33,41

TABLE 1. (continued)

No.	Compound	Formula	Lit. refs. Preparation	Lit. refs. M.s.	Permethylation products: Δ Ac groups	Δ Me groups	Interpretation	Lit. refs. Preparation	Lit. refs. M.s.
4	N^6-Acetyl-2′,3′,5′-tri-*O*-acetylcytidine		34	—	0	+1	N^6-Me derivative formed[b]	cf. 17	—
5	L-Pyrrolidonecarboxylic acid methyl ester[a]		8	8	—	+1[a]	*N*-CD_3 derivative formed; $-COOCH_3$ — $-COOCD_3$[a]	8	8
6	*N*-Benzyloxycarbonyl-3-chloro-L-alanine benzyl ester		—	—	—	+1	*N*-Me derivative formed; HCl eliminated to give 2-aminoacrylic acid residue[b]	—	—
7	DL-5-Phenylhydantoin		—	18,19,20	—	+3	1,3,5-Trimethyl derivative formed[b]	35	—
8	DL-5-(*p*-Hydroxyphenyl)hydantoin		16	cf. 16	—	+4	5-(*p*-Methoxyphenyl)-1,3,5-trimethylhydantoin formed[b]	—	—
9	N^1-Acetyl-DL-5-(*p*-acetoxyphenyl) hydantoin		—	—	0	+1	N^3-Me derivative formed[b]	—	—
10	*O,O′*-Diacetylcaffeic acid		36	—	0, −1, −2	+1, +2, +3	Methyl esters formed with partial deacetylative methylation of both phenolic groups; partial *trans* → *cis* isomerization[b]	—	cf. 21
11	Chlorogenic acid		—	—	—	+6 (+7)	Methyl ester of *O*-pentamethyl derivative formed (also slight *C*-methylation)	37,38	—
12	Penta-acetylchlorogenic acid		39	—	0 to −5	+1 to +7	Methyl ester formed with slight accompanying *C*-methylation; deacetylative methylation[b]	—	—
13	Tetra-acetylcaffeoylputrescine	 OR 	3,21[c]	21	−3	+4	*N*-(*O,O′*-Dimethylcaffeoyl)-*N′*-acetyl-*N,N′*-dimethyl-putrescine formed[b]	—	—

[a] Perdeuteriomethylation.
[b] See Discussion.
[c] E. I. Mbadiwe, unpublished work.

TABLE 2. Mass spectra (Ten most intense ions in decreasing order of intensity)
(P after compound number denotes permethylation product)

												M
1	*m/e*	138	156	198	111	110	153	180	139	240	171	360
	Rel. int.	100	66	64	54	43	36	24	21	21	20	0.1
1P	*m/e*	153	111	152	171	170	212	213	195	57	110	374
	Rel. int.	100	77	67	40	39	34	30	29	17	15	—
4	*m/e*	139	97	193	259	112	154	69	85	127	157	411
	Rel. int.	100	84	54	44	44	43	37	26	15	13	0.3
4P	*m/e*	139	259	97	152	69	174	115	126	168	207	425
	Rel. int.	100	69	65	32	28	23	20	19	15	14	3·0
6	*m/e*	91	107	92	65	108	79	39	77	51	89	347
	Rel. int.	100	16	8	8	5	4	4	4	3	2	0.01
6P	(Important ions		326	282	234	190	158	91)				
7	*m/e*	176	104	105	133	77	78	51	148	177	50	176
	Rel. int.	100	97	79	37	18	17	15	8	8	6	100
7P	*m/e*	203	118	218	56	204	77	91	51	119	132	218
	Rel. int.	100	67	27	24	20	20	9	8	8	7	27.0
8	*m/e*	120	121	192	65	39	164	163	92	93	94	192
	Rel. int.	100	63	55	21	15	14	10	7	7	6	55.0
8P	*m/e*	233	148	248	56	234	28	133	63	149	77	248
	Rel. int.	100	75	28	18	15	13	11	10	9	9	28.0
9	*m/e*	192	234	120	163	148	191	121	193	65	235	276
	Rel. int.	100	84	57	39	38	26	21	15	15	12	10.0
9P	*m/e*	206	248	205	290	120	148	249	207	163	121	290
	Rel. int.	100	83	32	28	23	16	11	11	9	8	28.0
10	*m/e*	60	180	222	136	181	57	55	69	134	51	264
	Rel. int.	100	56	12	7	6	5	4	4	4	4	2.5
10P	Mixture of 3 compounds[a]											—
11	*m/e*	163	180	162	134	43	44	89	29	135	111	354
	Rel. int.	100	82	64	26	24	19	18	17	16	14	14.0
11P	*m/e*	191	171	208	139	438	75	222	192	114	164	438
	Rel. int.	100	69	49	42	34	29	13	13	10	10	34.0
12	*m/e*	43	162	163	180	480	45	420	42	134	205	564
	Rel. int.	100	91	53	34	26	17	17	17	16	14	1.3
12P	Mixture of 5 compounds[a]											—
13	*m/e*	292	163	131	70	113	112	134	114	162	233	418
	Rel. int.	100	81	69	59	52	50	36	24	24	16	0.6
13P	*m/e*	191	84	157	57	137	192	163	55	348	71	348
	Rel. int.	100	48	46	26	24	22	20	15	14	13	14.0

[a] See Discussion.

the permethylation products have been stored by us and can be made available as required.

Discussion

The compounds studied were mostly not selected specifically for testing the questions under discussion but had presented themselves in the course of other researches. Our conclusions are to this extent tentative. Further study by g.c. has proved helpful where permethylation has led to multiple products.

Features of the mass spectra (Table 2)

Ions from the reaction products having 28 mass units less than the molecular ion or the higher fragments from the starting material proved to be valuable indicators of deacetylative methylation [M − 42 + 14≡M − 28].

1. Although the mass spectrum after permethylation (**1P**) only displays a very weak molecular ion (0.1 %), it is clear from the +14 mass unit shifts of major ions that methylation has occurred at only one position, presumably by esterification of the free carboxyl group.

4. Three sequential losses of 59 or 60 mass units from the molecular ion and the inter-ring fission process (*m/e* 259) characterize this class of compounds. Accurate mass measurements were used to confirm the processes. The molecular ion has Rel. int. 0.3 %. In

the permethylated product (**4P**), the molecular ion at *m/e* 425 and an [M − 15] ion at *m/e* 410 verify the presence of one additional Me group. Three sequential losses of 59 or 60 from the molecular ion and the ion at *m/e* 259 confirm that no methylation occurred on the acetylated sugar moiety. Methylation at N-6 was expected and can be assumed to have occurred (but see Lit.[17]).

6. The molecular ion region contains ions at *m/e* 347, 348, 349 and 350 (Rel. int. 0.01, 0.006, 0.004 and 0.002%, respectively). The indications are, that apart from the expected Cl isotope pattern, there is an [M + 1] process at 30% of M. Cl isotope patterns are associated with several fragment ions in the spectrum. The molecular ion expected for *N*-methylation of **6** would occur at *m/e* 361. However, the highest ion detected in the spectrum of **6P** was at *m/e* 326. This could be a [M − Cl] fragment. A more likely explanation is that HCl was abstracted during methylation to give the corresponding 2-aminoacrylic acid derivative and that an ion-molecule process (as with **6**) gives rise to an [M + 1] ion at *m/e* 326. This view is supported by the absence of Cl isotope patterns among the fragment ions. Regardless of doubt about the fate of the Cl atom, there is no doubt that neither of the benzyl ester linkages was rearranged (cf. Lit.[47]).

7. The fragmentation pattern of this compound has been studied in depth; mass measurements and complete metastable pathways have been elucidated (unpublished work by J. E. and R. S. (cf. Lit.[16]; see also Lit.[18,19,20]). The main decomposition pathway is initiated by loss of –HCNO from the molecular ion (*m/e* 176 → 133), followed by loss of –CO (→ *m/e* 105) and the loss of a proton to give *m/e* 104 (ϕ—C≡$\overset{+}{N}$H). On permethylation (**7P**), the addition of three methyl groups is evidenced by a molecular ion at *m/e* 218 (26.5%). The [M-15] ion at *m/e* 203 (100%) can be deduced[20] to be caused by the facile loss of methyl from C-5 (see also Lit.[19], Fig. 13). The next loss from this ion is the elimination of –$CONCH_3CO$, leaving the ion [ϕ—C≡$\overset{+}{N}CH_3$] at *m/e* 118 (67%). Together, these processes adequately establish the positions of the methyl substituents.

8P. The [M − 15] ion at *m/e* 233 (100%) again indicates the lability of the methyl group at C-5 of this 1,3,5-trimethylhydantoin.

9P. A molecular ion at *m/e* 290 indicates that only one methyl group has been added, whereas, from the behaviour of **7** and **8**, two could have been expected. The spectrum does not contain a [M − 15] ion, so we infer that the N^3-monomethyl derivative was formed and that no *C*-methylation occurred (see below, and above under Experimental).

10. The molecular ion at *m/e* 264 (2.5%) initiates two losses of ketene to *m/e* 222 and *m/e* 180. The base peak of the spectrum is *m/e* 60, which amounts to 35% of the total ion current. After permethylation (**10P**, not tabulated), it is obvious that partial deacetylative methylation has occurred and that three new compounds have resulted (mol. wts 278, 250 and 222, respectively). This was confirmed by g.c.-m.s. (1.5 m × 5 mm, 8% OV-1 glass column, temp. programme from 200 °C to 250 °C at 1 °C/min). Three major peaks (M = 222, 250 and 278) emerged successively. Each of these was preceded by a minor peak giving an essentially similar mass spectrum; these we attribute to partial rearrangement to the *cis* compounds during the permethylation.

11. This compound tends to dehydrate in the mass spectrometer, but by careful control of conditions a suitable spectrum was obtained. This was characterized by a molecular ion (Rel. int. 14%) and an [M − 18] process varying between Rel. int. 1 to 10% according to conditions. The caffeic acid fragment at *m/e* 180 (81%) loses —OH to form the base peak. The spectrum from the permethylation product (**11P**) is readily interpreted as the expected hexamethyl derivative. (An ion at [M + 14] having 1% of the intensity of M can be attributed to *C*-methylation). Partial rearrangement of the ester linkage, with formation of *O,O'*-dimethylcaffeic acid methyl ester, also occurred.

12. The molecular ion was present, and a series of losses of ketene were indicated. After permethylation (**12P**, not tabulated) the methyl ester resulted, with slight accompanying *C*-methylation. However, at least one, and perhaps all five of the acetyl groups, underwent deacetylative methylation. G.c. (as above, temp. programme 220 to 300 °C at 3 °C/min) showed at least five zones emerging above 280 °C.

13. Mass spectrometry (cf. Lit.[21]) has not satisfactorily settled which of the two structures given in Table 1 is correct. However, after permethylation (**13P**), a molecular ion at *m/e* 348 indicated that three of the four acetyl groups had been replaced by methyl. It is clear, from the occurrence of a fragment at *m/e* 191 as base peak, that both phenolic acetyls have been replaced by methyl groups. The complementary ion (*m/e* 157) loses 73 mass units ($\overset{+}{N}HCH_3COCH_3$) to give *m/e* 84 [$CH_2$=$CHCH_2CH$=$\overset{+}{N}HCH_3$] (cf. Lit.[22]).

The chemical reactions

As to ester rearrangement, carboxylic esters of methanol (**5** and ascorbalamic acid derivatives[8]) underwent complete rearrangement. Ester linkages to carbohydrates and related compounds seemed to remain largely intact (**1** to **4**, **11**, **12**). Previous studies on the stability of *O*-acyl groups in carbohydrates to methylating conditions are conflicting. Lindberg and colleagues found stability under the Kuhn conditions[40] but stated,[41] without detailed examples, that they were unstable under the Hakomori conditions (cf. Lit.[42]). Benzyl ester linkages (**6**) remained intact. The elimination of HCl from this chloro compound should be noted. In the *O*-acetylated monophenol studied (**9**), the ester linkage remained intact. This on the whole suggests that, on permethylation of peptide derivatives, deacetylative methylation of *O*-acetyl groups on residues of serine, threonine, hydroxylysine, hydroxyproline and

tyrosine should not occur. With all the *O*-acetylated polyphenols studied (**10**, **12**, **13**), at least partial deacetylative methylation took place. All were caffeic acid derivatives.

In general, *N*-acyl groups remained in position (**2** to **4**, **6**, **9**, **13**). However, the 'extra' acetyl group in the *N*-diacyl compound **13** was replaced by *N*-methyl.

N-methylation took place at all –CONH– groups as expected, except in **2**. As normal *N*-methylation took place in **3** (cf. Lit.[41]) some kind of intramolecular bonding of the –CONH– group of **2** to a contiguous *O*-acetyl group seems a more reasonable explanation of its inertness than does steric hindrance, the explanation favoured by Gorin,[43] who reports several similar failures of *N*-methylation (cf. Lit.[44]).

Substantial *C*-methylation was encountered only in **7** and **8**. These can be regarded as derivatives of 2-phenylacetic acid, which added three methyl groups under our permethylation conditions, presumably to give 2-methyl-2-phenylpropionic acid methyl ester (cf. Lit.[23]). The *C*-methylation must have taken place at C-5 of the hydantoin ring (see above). Acetylation at *N*-1 (**9**) prevented this *C*-methylation.

So far we have noted no differences in the outcome of permethylations in dimethyl sulphoxide conducted with potassium *t*-butoxide rather than with sodium hydride. This is not surprising, as Brauman *et al.*[24] consider that, on dissolution of the former in dimethyl sulphoxide, a substantial proportion of $CH_3SOCH_2^-$ anion occurs in equilibrium with the *t*-butoxyl anion. Dissolution of NaH is generally held to yield one molecular proportion of the carbanion.

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