Note

Improved derivative of 6-keto-prostaglandin F_{1z} for gas chromatographic–mass spectrometric analysis

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We describe here the properties and facile preparation of an improved derivative (III) of 6-keto-prostaglandin F_{1z} (6-keto-PGF_{1z}, II), the stable hydrolysis product of prostacyclin (I). The previously reported[1] methylester–methoxime–tris-trimethylsilyl (TMS) derivative (IV) has the serious disadvantages of susceptibility to hydrolysis by traces of moisture and a mass spectrum which is less than optimum for selected ion monitoring (SIM). The mass spectrum is deficient in high mass ions of high intensity for optimal quantitation by SIM. The improved derivative, a methyl ester–methoxime–tris-*tert.*-butyl dimethylsilyl ether (MeMOTBDMS, III), is easily prepared using a new silylating reagent, N-(*tert.*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), which has not been commercially available,

I

II

III R = TBDMS
IV R = TMS
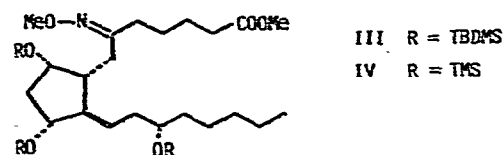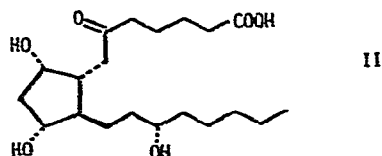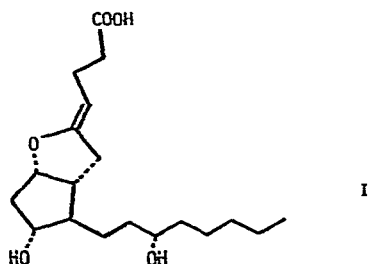
but is readily synthesized[2,*]. TBDMS derivatives are sufficiently stable that excess reagent and solvent may safely be evaporated so the derivative may be reconstituted in a minimum volume of solvent for improved sensitivity and reduced contamination of instruments. Alternatively the derivative may be injected in solution with excess reagent. This is a marked improvement over use of the *tert.*-butyldimethylchlorosilane–imidazole–dimethyl formamide (DMF) reagent of Corey and Venkateswarlu[3] which must be removed through extraction or chromatography[4] prior to analysis. The other important advantage of the derivative is the abundance of high mass ions suitable for SIM in the mass spectrum. The gas chromatographic (GC) properties of the derivative also make it highly suitable for GC–mass spectrometric (MS) analysis.

MATERIALS AND METHODS

Reagents and instruments

Ethyl trifluoroacetate was obtained from Eastman, Rochester, NY, U.S.A.; anhydrous monomethylamine was from Matheson, East Rutherford, NJ, U.S.A. NaH dispersion, anhydrous tetrahydrofuran (THF) and *tert.*-butyldimethylchlorosilane were from Aldrich, Milwaukee, WI, U.S.A. Methoxy-amine HCl was obtained from Pierce, Rockford, IL, U.S.A. and GC-grade pyridine was from Regis, Morton Grove, IL, U.S.A. Water was glass distilled. 6-Keto-PGF$_{1z}$ was a gift from Upjohn, Kalamazoo, MI, U.S.A.

Evaporation of solvents from samples was accomplished with a Model SVC-100H Speed Vac Concentrator from Savant Instruments, Hicksville, NY, U.S.A. GC analyses were accomplished with a Varian 1400 instrument equipped with a flame ionization detector and a silanized glass column (2 m × 2 mm I.D.) packed with 2% OV-17 on Supelcoport. GC–MS analyses were performed on Finnigan 3200 and Finnigan MAT 212 instruments.

Synthesis of silylating reagent

The method of Kutchinski[2] is here described because it is not readily accessable in the literature.

Preparation of N-methyltrifluoroacetamide. A flask containing ethyl trifluoro-acetate (29.7 g, 0.21 mole) was weighed and cooled to 0°C with dry ice. Anhydrous monomethylamine was bubbled through the ester while the temperature was main-tained at 0°C until the mass increase was 10.9 g (0.35 mole monomethylamine). The mixture was clear and colorless after standing at room temperature overnight. The product mixture was distilled; material boiling at 156–157°C was collected and crys-tallized upon cooling, m.p. 50–51°C, lit. 50–51°C[2], yield 78% of theoretical.

Preparation of N-(tert.-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). Baked glassware (250-ml three-neck flask, condensor, and addition funnel) was assembled and flushed with nitrogen. NaH dispersion (50%, 3.8 g, 0.078 mole) was washed with three 12-ml volumes of dry hexane. Anhydrous THF (78 ml) was added to the NaH under nitrogen and N-methyltrifluoroacetamide (10 g, 0.078

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* The reagent has recently been made available commercially by Regis Chemical Co., Morton Grove, IL, U.S.A.

mole, in 10 ml of anhydrous THF) was added. The mixture was stirred under reflux and nitrogen for 4 h before adding *tert.*-butyldimethylchlorosilane (11.8 g, 0.078 mole, in 6 ml of THF). Reflux under nitrogen was continued for 24 h. Solvents were removed through rotary evaporation, and the residue was stored overnight in a capped flask. The residue was distilled and the fraction boiling at 174–182°C collected. Infrared (IR) and nuclear magnetic resonance (NMR) spectra were in agreement with literature[2] spectra and amounted to 32 % of the theoretical yield. The reagent is stored at room temperature in a desiccator.

Derivatization of 6-keto-PGF$_{1x}$

Methoximation. Methoxyamine HCl (0.1 ml of 0.025 g reagent/ml dry GC-grade pyridine) was added to a residue of the prostaglandin in a silanized centrifuge tube. After capping (PTFE-lined cap) and vortexing, the mixture was either warmed at 38°C for 1 h or left at room temperature overnight. The pyridine was then evaporated with nitrogen while the tube was partially immersed in warm water. Water (0.5 ml) was added, the mixture was acidified with dilute formic acid to pH 3.5 and then extracted twice through vortexing with 1-ml volumes of diethyl ether. The combined extracts were washed with 1 ml of water and the ether was evaporated in a vacuum centrifuge.

Esterification. Etherial diazomethane (0.2 ml *ca.* 0.6 *M*) was added to the methoxime. Dissolution of the methoxime was assured through vortexing. After 5 min excess reagent was dispersed with a stream of nitrogen, and the etherial solution was transferred by pipette to a 1-ml Reactivial®. A small ether wash (0.1 ml) was also transferred to the vial and the ether was evaporated under nitrogen.

Silylation. DMF (25 μl) and MTBSTFA (25 μl) were next added to the vial and complete dissolution was assured through vortexing. The capped vials were heated at 60°C for 1 h, then the solvent and reagent were evaporated with a stream of nitrogen. The residue was reconstituted in 10 μl hexane. Alternatively the reaction mixture could be injected directly.

Instrumental parameters. For GC analysis the carrier gas (nitrogen) flow-rate was 30 ml/min. Injector temperature, 270°C; detector temperature, 300°C; column temperature, 260°C. GC–MS analysis was performed with a helium flow-rate of 12 ml/min and the following parameters: electron energy, 70 eV; injector temperature, 270°C; column temperature, 260°C; separator temperature, 270°C.

RESULTS AND DISCUSSION

In recent years much attention has been devoted to the optimization of the analysis of 6-keto-PGF$_{1x}$. The result has been improvements in the analytical procedure. Most recently Hensby *et al.*[5] have compared the sensitivity and specificity of GC–MS and radioimmunoassay (RIA), and found RIA to be more sensitive but more subject to interferences. They state the importance of the validation of an RIA for a particular prostaglandin by GC–MS, a technique which is both highly specific and sensitive. We now offer a further improvement in the derivatization of 6-keto-PGF$_{1x}$ for analysis by GC–MS.

The original derivatization published by Pace-Asciak[1] involved esterification followed by methoximation and trimethylsilylation to yield a methyl ester–meth-

oxime–tris-TMS (MeMOTMS) derivative. Papers with attention to various details and variations in the procedure were subsequently published[6,7]. A significant variation concerned the order of esterification and methoxima: on. Like Pace-Asciak, most workers have proceeded to first esterify and then form the methoxime; however, Clayes et al.[6] have reported a five-fold increase in derivative yield at the 100-ng level by first executing the methoximation. We have adopted this sequence in our procedure.

The necessarily huge excess of methoxyamine HCl has been dealt with differently by several authors. Frölich[7] in a general derivatization procedure suggests extraction of the derivative with ether from excess solid reagent after evaporation of pyridine. Oliw et al.[8] apparently proceeded to esterify and silylate in the presence of excess reagent. We have found these methods to be less than optimum, and prefer evaporation of the pyridine and extraction of the methoxime with ether from an acidified aqueous solution of methoxime and excess reagent. Evaporation of the water-washed ether extracts is accomplished with a centrifugal vacuum evaporator which concentrates the derivative residue in the bottom of a silanized centrifuge tube.

For esterification, the use of freshly prepared etherial diazomethane is specified by many authors, and many use a mixture of methanol–etherial diazomethane. We find it effective and much more convenient to use only an etherial solution of diazomethane prepared in advance and stored at $-20°C$. The reagent keeps well for weeks under these conditions.

In recent years attention has been focused on improving the mass spectra of prostaglandins for quantitative analysis through SIM. Thus, Watson and Sweetman[9] prepared methyl ester TBDMS derivatives of PGA and PGB, and reported mass
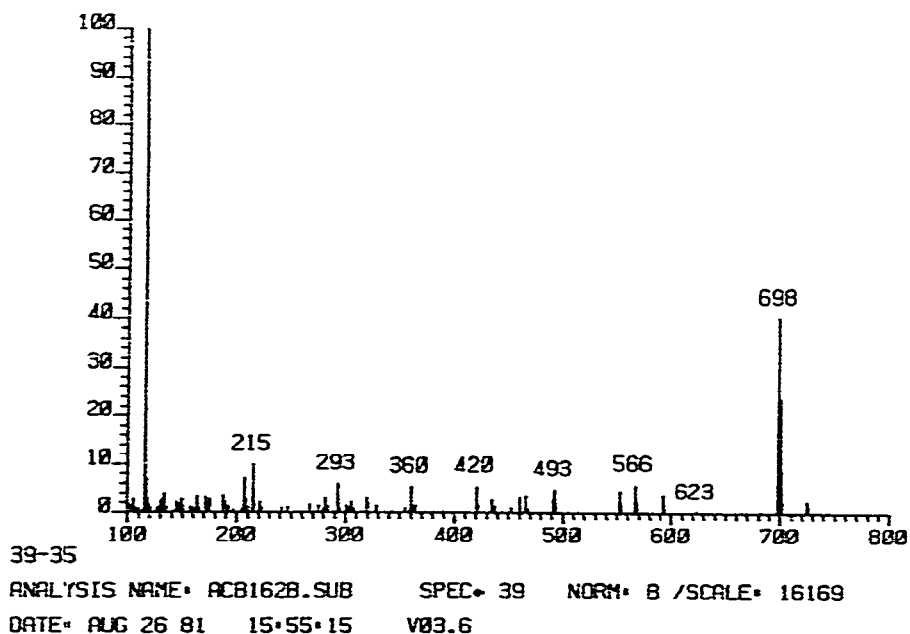

39-35

ANALYSIS NAME: ACB162B.SUB   SPEC: 39   NORM: B /SCALE: 16169
DATE: AUG 26 81   15:55:15   V03.6

Fig. 1. Mass spectrum of MeMOTBDMS derivative of 6-keto-PGF$_{1α}$.

spectra with abundant molecular ions and base peaks at M − 57 from loss of the *tert.*-butyl group. Gaskell and Pike[10] have also emphasized the advantages of TBDMS ethers in their recent paper on the GC–MS of methyloxime-TBDMS derivatives of androstanolones. For silylation all of these authors employed the *tert.*-butyldimethylchlorosilane–imidazole reagent, which must be removed through extraction or chromatography prior to analysis*.

Pace-Asciak[11] reported that the MeMOTMS derivative of 6-keto-PGF$_{12}$ had a "multiplicity of ions of high intensity in the high end of the mass spectrum, providing excellent potential for use in mass fragmentography." These results were obtained with a Varian MAT CH-5, a magnetic sector instrument. Unfortunately such multiplicities of high-intensity high-mass ions are often not seen, especially with quadrupole instruments which may discriminate against the high-mass end of the spectrum.

One solution to this problem, that of Morita and co-workers[12,13], is the use of chemical-ionization (CI) mass spectrometry. These authors compared ammonia,

TABLE I

MASS SPECTRA OF MeMOTMS[14] AND MeMOTBDMS (III) DERIVATIVES OF 6-KETO-PGF$_{12}$
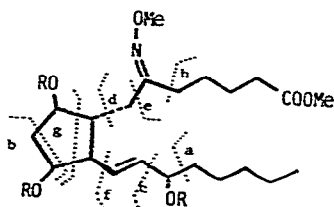
Numbers shown are mass numbers (m/z) with relative abundances given in parentheses.

Ion assignment	MeMOTMS (IV)		MeMOTBDMS (III)	
[M]$^{+ \cdot}$	629	(8)	755 (not observed)	
[M − Me]$^+$	614	(6)	—	
[M − *tert.*-Bu]$^+$	—		698	(40)
[M − OMe]$^+$	598	(52)	724	(2)
[M − HOR]$^{+ \cdot}$	539	(13)	623	(0.3)
[M − OMe − HOR]$^+$	508	(64)	592	(4)
[M − OMe − b]$^+$	482	(5)	566	(6)
[M − HOR − a]$^+$	468	(34)	552	(5)
[M − d]$^+$	443	(6)	569	(0.7)
[M − OMe − HOR − b]$^+$	392	(10)	434	(3)
[M − 2(HOR) − a]$^+$	378	(100)	420	(5)
[M − d − HOR]$^+$	353	(28)	437	(1)
[M − OMe − 3(HOR)]$^+$	328	(25)	328	(1)
[M − 2(HOR) − c]$^+$	277	(17)	319	(3)
[M − 2(HOR) − d]$^+$	263	(17)	305	(2)
[g]$^+$	217	(24)	301	(1)
[f]$^+$	199	(25)	241	(1)
[d + H]$^{+ \cdot}$	187	(26)	187	(4)
[c]$^+$	173	(61)	215	(10)
[h]$^+$	115	(49)	115	(100)
[TBDMS]$^+$	—		115	(100)
[TMS]	73	($\gg$ 100)	—	

methane, and isobutane as reagent gases and found ammonia to be the superior reagent. With ammonia the quasimolecular ion (QM^+, m/z 630) was weak; but a base peak at m/z 540 ($QM^+ - TMSOH$) was used for quantitative mass fragmentography with practically no interference from other endogenous biological substances. These results point out the advantage of monitoring high-intensity high-mass ions. However, since many laboratories are not equipped for GC–CI–MS, an alternative solution would be useful. We offer an easily prepared new derivative as a means of obtaining interference-free high-intensity high-mass ions for quantitative analysis of 6-keto-PGF_{1z} through SIM.

The mass spectral fragmentation of the new derivative of 6-keto-PGF_{1z} closely parallels that of the MeMOTMS derivative which has been well characterized by Cockerill *et al.*[1+]. The mass spectrum of the new derivative and a tabular comparison of its fragmentation with that of the MeMOTMS derivative appear in Fig. 1 and Table I, respectively. It should be noted that the data in Table I for the relative intensities of the high-mass ions from IV appear high, but this is due to normalization on the m/z 378 peak rather than the most intense m/z 73 peak. If the analogous peak (m/z 115) from III were omitted, the $M - 57$ peak (m/z 698) would become the base peak. It can be seen from Table I that there are several high-intensity high-mass ions in the mass spectrum of III in which the hydrogen atoms at C-3 and C-4 are retained. This is significant because it allows use of the 3,3,4,4,-$^2H_4$ analogue as an internal standard.

A further simplification of the derivatization of 6-keto-PGF_{1z} is the simultaneous esterification and ether formation through reaction of the methoxime with MTBSTFA to form the MO TBDMS ester TBDMS ether. However, we find the TBDMS ester to be inferior to the methyl ester for GC–MS analysis; GS retention time is longer and the higher mass of fragment ions may present problems for limited mass range instruments. Neither the methyl ester nor the TBDMS ester derivatives showed GC separation of the *syn*- and *anti*-methyloxime isomers.

In conclusion, this new derivative of 6-keto-PGF_{1z} is comparatively easily prepared as described and offers the advantages of hydrolytic stability and superior mass spectrometric properties for GC–MS–SIM quantitation.

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