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# IDENTIFICATION OF REACTION PRODUCTS FROM THE PYRIDINIUM DICHROMATE DERIVATIZATION OF PROSTAGLANDINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND DIRECT CHEMICAL IONIZATION MASS SPECTROMETRY

JO DOEHL*[a] and TYGE GREIBROKK
*Department of Chemistry, University of Oslo, P.O. Box 1033, Blindern, 0315 Oslo 3 (Norway)*
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## SUMMARY

The reaction products from the oxidation of prostaglandins with pyridinium dichromate have been identified by direct chemical ionization mass spectrometry of the underivatized compounds after separation by reversed-phase high-performance liquid chromatography and ultraviolet diode-array detection. The thermal influence on the reproducibility of the dehydration patterns of the mass spectra was studied. The main products from the prostaglandins $E_1$, $E_2$, $F_{1\alpha}$ and $F_{2\alpha}$ were the corresponding 15-oxo derivatives. Minor amounts of the 9,11,15-trioxoprostaglandin were formed from PGE, while the oxidation of PGF was less selective, yielding additional dioxo derivatives. Addition of water to the reagent reduced the reactivity, but increased the selectivity in favour of the formation of 15-oxo-PGF during the oxidation of PGF.

## INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become a widespread technique for the separation of prostaglandins (PGs)[b]. After separation, the isolated double bonds of non-derivatized PGs have been detected by UV spectrophotometry at low wavelengths (190–215 nm)[1–8]. However, derivatization is usually preferred in order to increase the sensitivity and selectivity. This includes fluorescent labelling of PGs with 4-bromomethyl-7-methoxycoumarin[9,10], 7-acetoxy-4-bromomethylcoumarin[11,12], 7-[(chlorocarbonyl)methoxy]coumarin[13], aromatic isocyanates[10], anthroylnitrile[14], *p*-(9-anthroyloxy)phenacylbromide[15,16] and anthryldiazomethane[17–19]. Recently, detection limits of 10–30 fmol (PGE, PGF; signal-to-noise ratio, S/N = 5, on column) have been obtained with 3-bromomethyl-6,7-

[a] Present address: Center for Industrial Research, P.O. Box 124, Blindern, 0314 Oslo 3, Norway.
[b] In this text, prostaglandins are collectively referred to as PGs, ($\Delta^{13,14}$)-prostaglandins as $PG_1$ and ($\Delta^{13,14}$,$\Delta^{5,6}$)-prostaglandins as $PG_2$, while PG without a subscript denotes $PG_1$ *and* $PG_2$ (PGE = $PGE_1$ *and* $PGE_2$).

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dimethoxy-1-methyl-2(1H)-quinoxalinone[20] and 1-pyrenyldiazomethane[21]. The formation of UV-absorbing substituted phenacyl esters[22–26], benzyloximes[27], naphthacyl esters[28–30] and benzyl esters[31] as well as the base catalysed conversion of E and A prostaglandins (PGE and PGA) into PGB ($\lambda_{max}$ = 278 nm)[32–36] have been described. Detection limits of 140 fmol (S/N = 5, on column) have been achieved with 2,4-dimethoxyanilides and electrochemical detection[37].

We have previously reported a rapid UV derivatization procedure in which E-type PGs (PGEs) are selectively oxidized to their 15-oxo derivatives by pyridinium dichromate (PDC) in acetonitrile with detection limits of 0.14 pmol (S/N = 2, on column)[38,39]. In this paper the derivatization is further characterized with respect to the reactivity, selectivity and identity of the reaction products.

## EXPERIMENTAL

### *Chemicals*

All PGs were obtained from Sigma (St. Louis, MO, U.S.A.), PDC (98%) from Aldrich (Milwaukee, WI, U.S.A.) and formic acid (p.a.) from Merck (Darmstadt, F.R.G. ). Acetonitrile, ethyl acetate and dichloromethane were HPLC grade from Rathburn (Walkerburn, U.K.). Deionized water was distilled once. PG standards were dissolved in acetonitrile ($2.8 \cdot 10^{-4} M$) and stored at −20°C.

### *HPLC equipment*

The HPLC instrumentation consisted of a solvent-delivery system (Waters Model 590), a valve loop injector (Waters U6K) connected to a $C_{18}$ column [Brownlee MPLC™, 200 mm × 2.1 mm, 5-μm $C_{18}$ silica (Spheri-5) or Perkin-Elmer, 33 mm × 4.6 mm, 3-μm $C_{18}$ silica (Pecosphere)] and a diode-array UV detector (Hewlett-Packard 1040A). Water, acetonitrile (HPLC grade S) and formic acid were used as the mobile phase.

### *Mass spectrometry*

Direct chemical ionization mass spectrometry (DCI-MS) was performed on a double focusing mass spectrometer (JEOL JMS-DX 303) with methane or isobutane (AGA Specialgas) as the reagent gas. Measurement conditions: accelerating voltage, 3 kV; ionizing voltage, 200 eV; ionizing current, 300 μA; electron multiplier voltage, 1.5 kV and chamber temperature 210°C.

### *Derivatization of PGs*

Unless stated otherwise, PGs were oxidized by PDC in acetonitrile, diluted in water and extracted with ethyl acetate to remove the excess of reagent as previously described[39].

## RESULTS AND DISCUSSION

### *Identification of reaction products*

Oxidation of PGE under the conditions stated above resulted in one major product in high yields, whereas the oxidation of PGF resulted in a more complicated pattern. This is illustrated in Figs. 1–4, in which the three-dimensional isoabsorbance

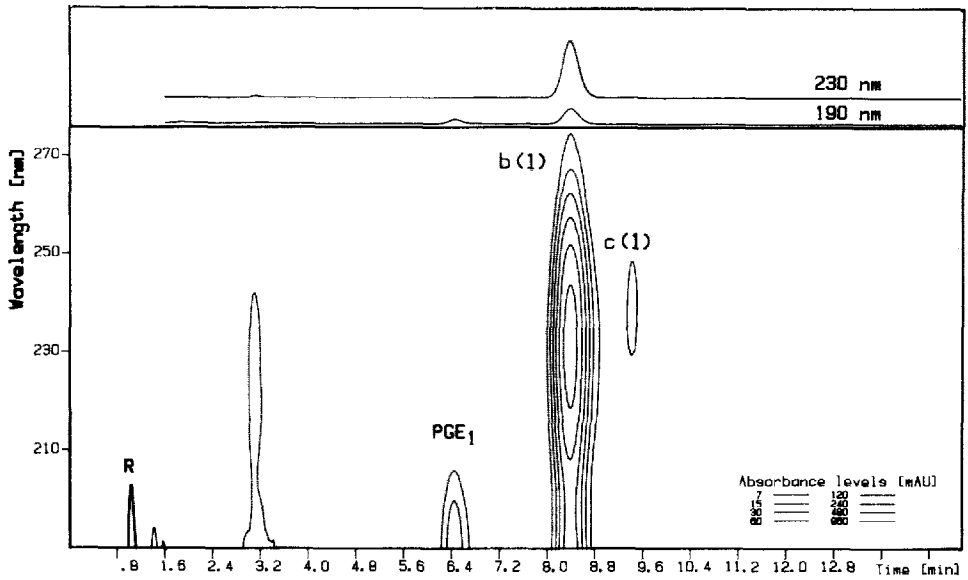



Fig. 1. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of $PGE_1$. Upper section: single-channel chromatograms; UV detection at 190 and 230 nm. Lower section: three-dimensional isoabsorbance diagram; UV diode-array detection. Column: $C_{18}$ (Brownlee, 5-$\mu$m RP-18 silica, 200 mm × 2.1 mm I.D.). Mobile phase: acetonitrile–5 m*M* aqueous formic acid (36:64) at 0.4 ml/min. R = Reagent peak (PDC). For other structures see Fig. 7.

$C_{18}$ reversed-phase chromatograms of the reaction mixtures of each $PGE_1$, $PGE_2$, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are shown. The PGE chromatogram (Figs. 1 and 2) are dominated by compound b[a] and a minor contribution from the later-eluting compound c. The PGF chromatograms (Figs. 3 and 4) reveal three major components, a, b and d, under similar reaction conditions. Compound b from PGF will be coeluted with compound b from the corresponding PGE. The spectroscopic properties of all compounds a–d (Figs. 1–4, Table I) are clearly indicative of the presence of a conjugated enone chromophore.

To obtain further structural information, the reaction products of each PG were subjected to mass spectrometric analysis after isolation by fraction collection and extraction from the acidic aqueous mobile phase by dichloromethane (1:1, v/v). Besides being a convenient solvent for application on the solid sample probe, the concentrated dichloromethane solution (0.5 ml reduced to 10 $\mu$l) resulted in far less chemical noise than did unpurified HPLC grade ethyl acetate. Underivatized PGs can be analysed by electron-impact ionization (EI-MS), resulting in numerous fragment ions and complex spectra[40–42] which obscure the information on molecular weights[43]. By exposing non-volatile compounds on a heated probe to an appropriate ionized gas, molecular weight information can be obtained from the resulting CI spectra. This is frequently referred to as "direct exposure", "desorption CI", "direct CI (DCI)", "in-beam" or "surface ionization"[43–45]. Hydroxylated compounds like

[a] Abbreviated PG derivative names without an index denote $PG_1$ *and* $PG_2$ [b = b(1) *and* b(2)]. See Fig. 7.

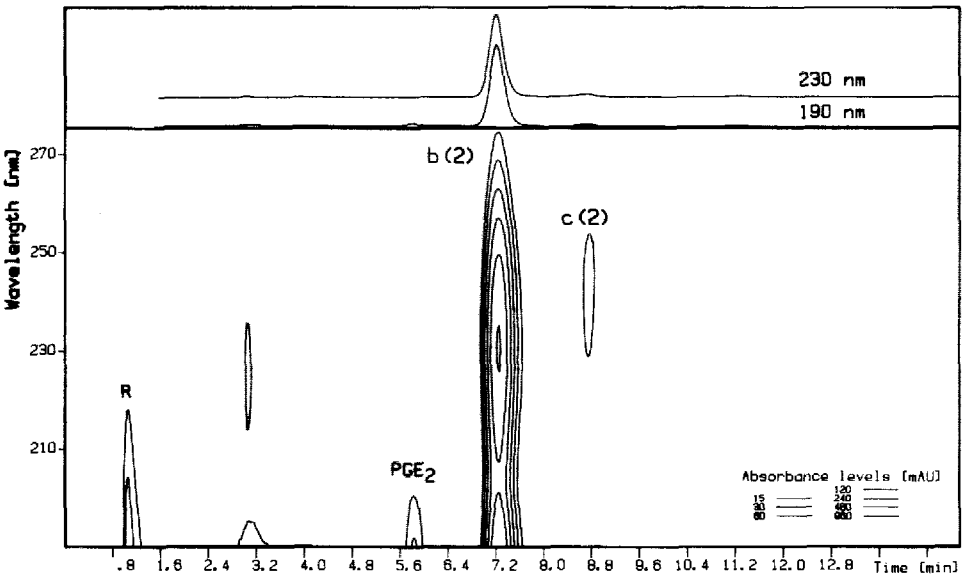



Fig. 2. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of $PGE_2$. Conditions as in Fig. 1.

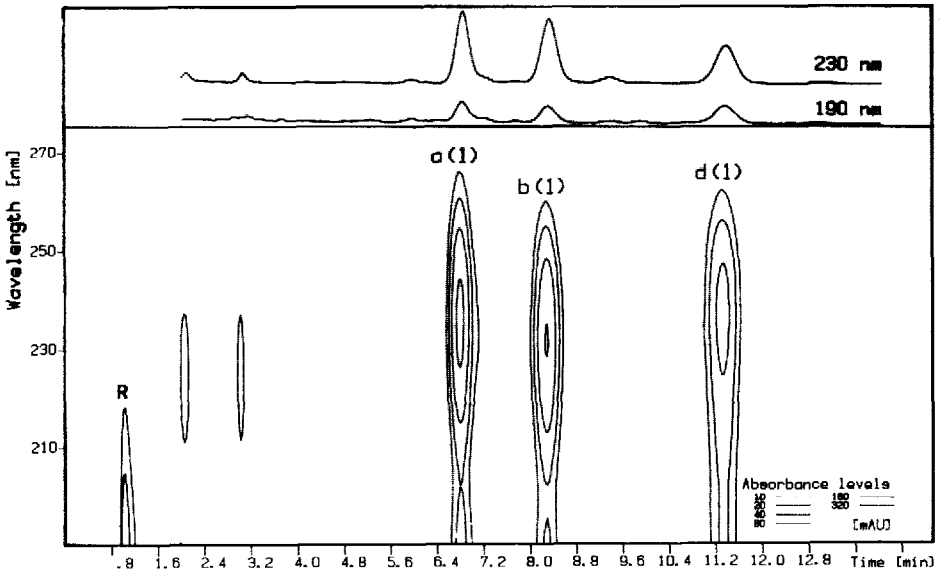



Fig. 3. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of $PGF_{1\alpha}$. Conditions as in Fig. 1.

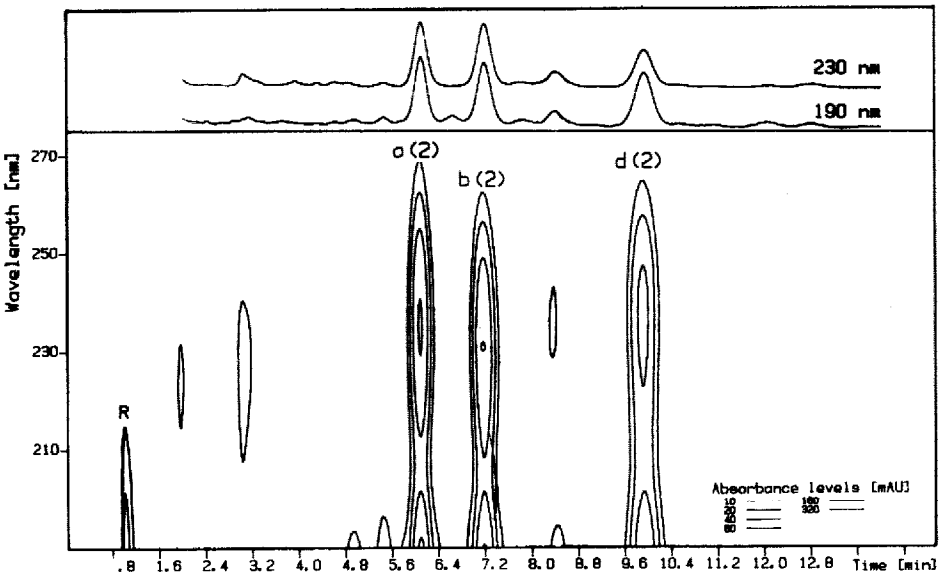



Fig. 4. Reversed-phase HPLC separation of the PDC–acetonitrile reaction mixture of $PGF_{2\alpha}$. Conditions as in Fig. 1.

PGs will undergo dehydration under protonating CI conditions. The extent of dehydration can be reduced by increasing the proton affinity of the reagent gas relative to the analyte, *e.g.*, replacing methane with ammonia[45]. A protonating reagent gas can, however, be advantageous with respect to the identification of polar substituents, since the number of water losses will reflect the number of hydroxyl and/or ketone functions in the molecule, as observed in thermospray MS of PGs[46]. The dehydration process is clearly demonstrated by the isobutane CI spectra of $PGF_{2\alpha}$ and $PGF_{1\alpha}$ (Fig. 5) where three consecutive water losses were observed, *m/z* ($PGF_{1\alpha}/PGF_{2\alpha}$) 339/337, 321/319 and 303/301, in addition to ions formed by elimination of $CO_2$. The possible combinations of these neutral losses are shown in Fig. 6. Ions $MH^+$ and $[M-H]^+$ of low abundances were also observed, *m/z* ($PGF_{1\alpha}/PGF_{2\alpha}$) 357/355 and 355/353, as was a series of dehydrated ions from the $[M+C_4H_9]^+$ adduct, *m/z* ($PGF_{1\alpha}/PGF_{2\alpha}$) 395/393, 377/375 and 359/357.

TABLE I

UV ABSORPTION MAXIMA IN ACETONITRILE–5 m*M* AQUEOUS FORMIC ACID OF PGs AFTER PDC OXIDATION

Abbreviated names refer to Figs. 1–4 and 7.

| | $\lambda_{max}$ (nm) |
|---|---|
| $PGE_1/PGE_2$ | <190/<190 |
| $PGF_1/PGF_2$ | <190/<190 |
| a(1)/a(2) | 234/234 |
| b(1)/b(2) | 230/230 |
| c(1)/c(2) | 242/242 |
| d(1)/d(2) | 234/234 |

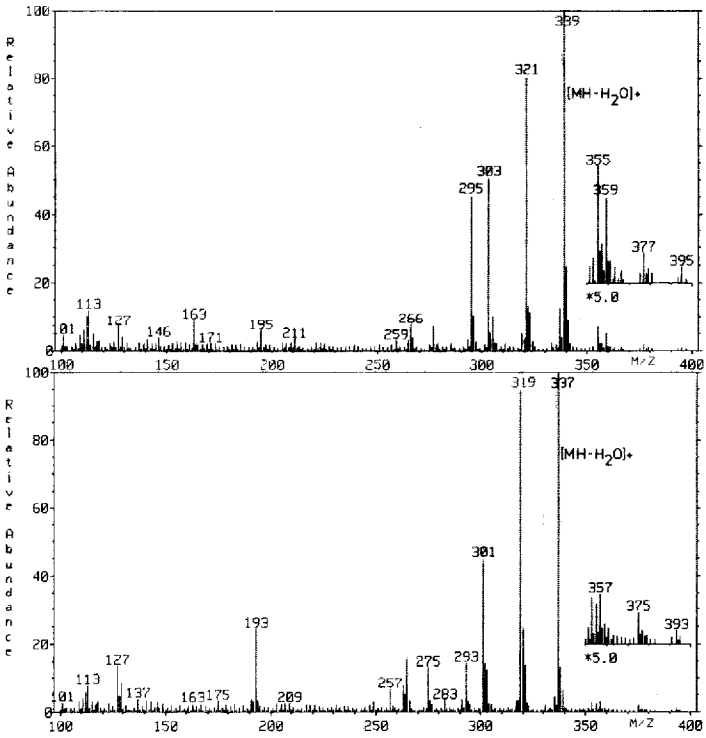



Fig. 5. Direct CI-MS (DCI-MS) of $PGF_{1\alpha}$ (MW 356) (upper spectrum) and $PGF_{2\alpha}$ (MW 354) (lower spectrum). Reagent gas: isobutane.

The partial isobutane DCI mass spectra of oxidized and non-oxidized PGs are compared in Table II. Spectra were obtained by heating a conventional quartz capillary solid sample probe from ambient temperature to 100°C in 6 s, keeping it isothermal for 10 s and finally raising the temperature to 200°C in 20–30 s. The ion source was kept at 210°C. As is seen from Table II, protonated molecules ($MH^+$) of the native PGs were low in abundance (1–4%) as compared to those for the oxidized PGs (7–100%). This is probably reflecting the presence of the allylic activated hydroxyl group at C-15 and/or a $\beta$-ketol (PGE) from which dehydration will easily occur.

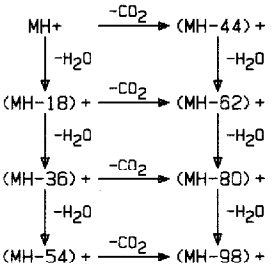



Fig. 6. Formation of fragment ions in DCI-MS of PGs by successive dehydration and decarboxylation.

The mass spectrum of each compound in the reaction sequence PGE->a->c (Table II) revealed a molecular weight reduction of 2 mass units and the disappearance of one hydroxyl group, *i.e.*, one hydroxyl group is oxidized, in each step. The same observation was made for the sequence PGF->a->b,d. Together with the UV spectroscopic and chromatographic data (Figs. 1–4, Table I), the structures in Fig. 7 have been assigned. The proposed structure of d(2) was confirmed by PDC oxidation of $PGD_2$ which yielded a compound with identical DCI mass spectrum, UV spectrum and HPLC retention time. The reversed-phase retention of compound c, eluted between the di oxo derivatives b and d, is not readily explained from its assigned tri oxo structure, suggesting a keto–enol conversion of the 9,13-dione to an hydroxyenone.

Although elimination of water was not observed exclusively from hydroxyl groups, but also from ketones, the latter is likely to happen less frequently, requiring a double proton transfer. With the exception of compound d, the data were in accordance with this assumption. After the assigned number of hydroxyl groups had been eliminated, there was a drastic reduction in the abundance of ions from continued dehydration (Table II). Assuming that the first dehydration step of compounds b and d is the formation of a $\beta$-enone in the cyclopentane ring, further elimination of water from d might be facilitated by the formation of an extended conjugated system fol-

TABLE II

PARTIAL ISOBUTANE DCI MASS SPECTRA OF PGs AND PRODUCTS FROM THE OXIDATION OF PGs WITH PDC

Abbreviated names (a–d) refer to corresponding components of the chromatograms in Figs. 1–4. The first and second entry in each column is the observed $m/z$ value. Column indices 1 and 2 denote $PG_1$ and $PG_2$ respectively. Other entries are intensities relative to the base peak (= 100). An asterisk (*) denotes fragments resulting from dehydration of other than hydroxyl functional groups. Upper section: $[MH-nH_2O]^+$. Lower section: $[MH-CO_2-nH_2O]^+$.

| | | *PG* | *Products* | | *PG* | *Products* | | |
|---|---|---|---|---|---|---|---|---|
| | | *PGE* ⟶ | *a* | *c* | *PGF* ⟶ | *a* | *b* | *d* |
| MH ($m/z$) | 1: | 355 | 353 | 351 | 357 | 355 | 353 | 353 |
| | 2: | 353 | 351 | 349 | 355 | 353 | 351 | 351 |
| $MH^+$ | 1: | 1 | 9 | 100 | 3 | 100 | 10 | 11 |
| | 2: | 1 | 8 | 100 | 4 | 100 | 8 | 7 |
| $[MH-18]^+$ | 1: | 36 | 100 | 7* | 100 | 88 | 100 | 100 |
| | 2: | 30 | 100 | 11* | 100 | 57 | 100 | 100 |
| $[MH-36]^+$ | 1: | 100 | 5* | 2* | 80 | 16 | 7* | 27* |
| | 2: | 100 | 6* | 1* | 95 | 19 | 6* | 23* |
| $[MH-54]^+$ | 1: | 7* | 1* | 1* | 50 | 1* | 1* | 1* |
| | 2: | 5* | 1* | 3* | 45 | 4* | 0* | 2* |
| $[MH-44]^+$ | 1: | 2 | 3 | 11 | 1 | 6 | 9 | 2 |
| | 2: | 1 | 4 | 10 | 2 | 5 | 1 | 3 |
| $[MH-62]^+$ | 1: | 10 | 11 | 2* | 45 | 8 | 14 | 29 |
| | 2: | 13 | 30 | 5* | 17 | 5 | 22 | 30 |
| $[MH-80]^+$ | 1: | 25 | 3* | 0* | 25 | 3 | 2* | 3* |
| | 2: | 27 | 2* | 1* | 10 | 3 | 0* | 4* |
| $[MH-98]^+$ | 1: | 2* | 1* | 2* | 3 | 0* | 1* | 0* |
| | 2: | 2* | 2* | 3* | 6 | 0* | 1* | 0* |

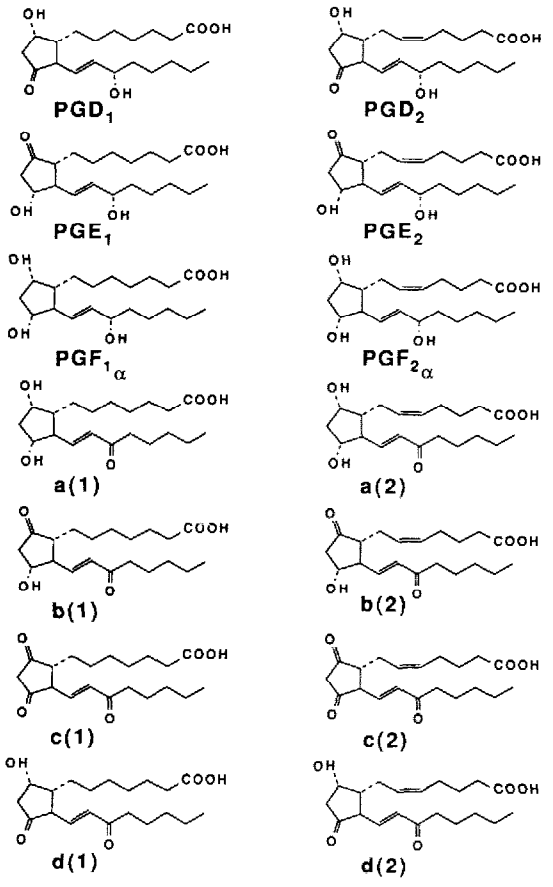



Fig. 7. PG structures and proposed structures of reaction products from the PDC oxidation of PGs.

lowing the 1,2-elimination of water from a protonated ketone at C-11, with possible contributions from the keto–enol tautomeric properties of the PGD C-11 keto group[47]. This may explain the difference in abundance between the $[MH-36]^+$ of b and d.

Relative ion abundances, in particular of the protonated molecules, varied both within and between experiments. In some cases, the variations between corresponding $PG_1$ and $PG_2$ were also larger than expected (Table II). In Fig. 8a is shown the variation in the relative ion abundances of the isobutane DCI spectrum through the total ion current (TIC) evaporation profile of $PGF_{1\alpha}$. The abundances of multi-dehydrated ions increased with time at the expense of $MH^+$ (not shown) and $[MH-H_2O]^+$, leading to a changeover in the base peak from $[MH-H_2O]^+$ to $(MH-2H_2O)^+$. Decarboxylation ions (*m/z* 295,277) were less influenced. The spectral variations of PGF were considerably reduced with methane as the reagent gas (Fig. 8b). Evidence for a possible thermal origin for these variations was provided by the work of Field[48], who found isobutane spectra of acetates to be strongly dependent on temperature, whereas methane spectra were less affected. Apparently, the

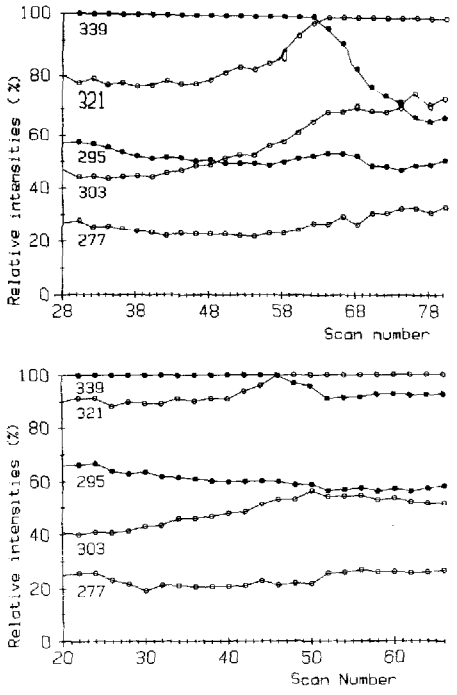



Fig. 8. Ion current profiles indicating the consistency of the DCI mass spectrum of $PGF_{1\alpha}$. Reagent gases: isobutane (upper spectrum) and methane (lower spectrum).

reduced exothermic nature of the isobutane protonation [proton affinity (PA) = 824 kJ/mol] compared to methane (PA = 546 kJ/mol) makes the fragmentation of the former more sensitive to variation in the internal energy prior to ionization. As previously questioned[45], an additional contribution to the spectral variations from thermal preionization dehydration cannot be excluded, since increased dehydration with time was observed even with methane CI. In particular, a time-dependent strong reduction of the ion $(MH-H_2O)^+$ was found in the CI spectra of PGE and $PGD_2$, independent of the choice of methane or isobutane as the reagent gas. Although the probe temperature was rapidly increased, the time needed for heat transfer to the quartz capillary and the sample may very well be responsible for the observed time-dependent abundance variations. Lowering the probe temperature somewhat reduced this effect, but resulted in reduced spectrum intensities due to broadening of the TIC profile, in particular of the more polar samples, *e.g.*, PGF. Below a probe temperature of 150°C the signal disappeared, as was the case below a source temperature of 150°C. Although the thermal effects will limit the reproducibility of ion abundances, the spectra can still be interpreted on a qualitative basis. In some cases, the DCI mass spectrum can even differentiate between positional isomers: the methane DCI spectrum of $PGE_2$ was readily distinguished from that of $PGD_2$ by the abundance of the ion $(MH-3H_2O)^+$ (*m/z* 299, Fig. 9), which was considerably higher with $PGE_2$ [relative abundance (R.A.) = 30%] than with $PGD_2$ (R.A. = 7%). This property did not change through the probe heating process. Isobutane as a reagent gas gave a much smaller difference.

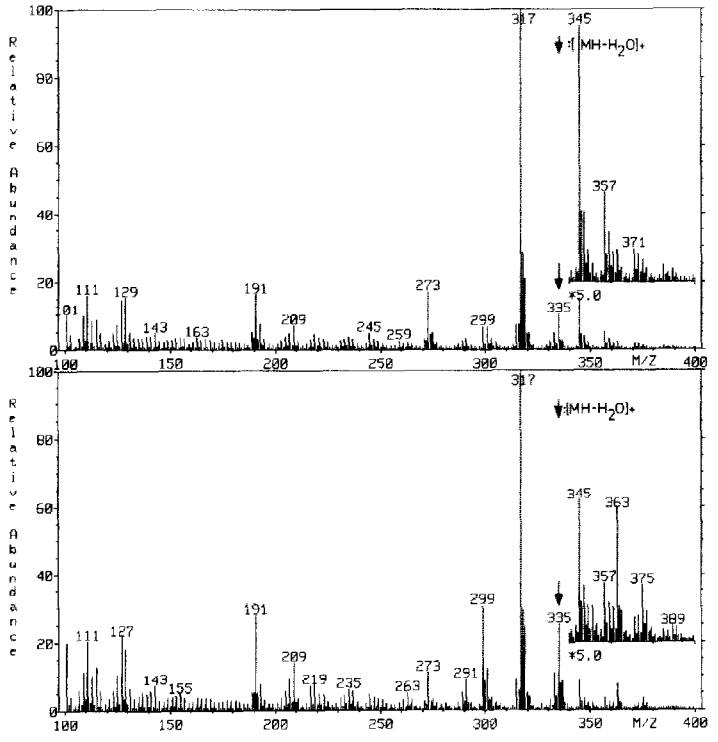



Fig. 9. DCI-MS of $PGD_2$ (MW 352) (upper spectrum) and $PGE_2$ (MW 352) (lower spectrum). Reagent gas: methane.

Increasing the solvent polarity will reduce the reactivity of PDC. To determine the influence of the solvent water content on the reactivity and selectivity, $PGF_{1\alpha}$ was oxidized in the presence of various concentrations of water. By direct injection of 1-$\mu$l aliquots of the reaction mixture on the short 3-$\mu$m particle column with a solvent flow-rate of 2.0 ml/min, the separation of all PGs was complete in less than 2 min, permitting a nearly real time monitoring of the oxidation, as shown in Figs. 10–12. According to the reaction kinetics, there is no need for extra drying of acetonitrile since a water content of 0.1% did not influence the reaction rate. On the other hand, a

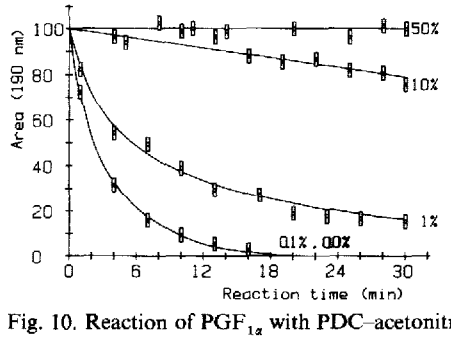
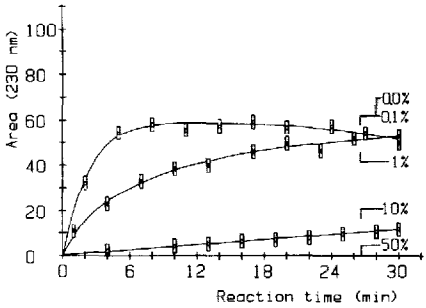



Fig. 10. Reaction of $PGF_{1\alpha}$ with PDC–acetonitrile in the presence of various concentrations of water.



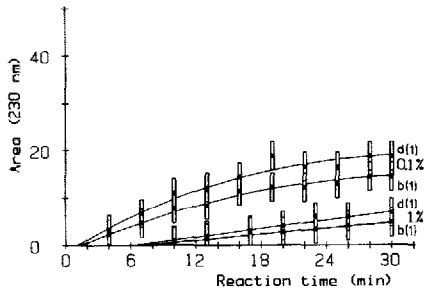




Fig. 11. Formation of the primary oxidation product a(1) from $PGF_{1\alpha}$ in the presence of various concentrations of water.

Fig. 12. Formation of the secondary oxidation products b(1) and d(1) from $PGF_{1\alpha}$ in the presence of various concentrations of water.

water content of 1% resulted in a selectivity change by reducing the formation of the by-products b and d (Fig. 12), although at the cost of reaction time (>30 min) (Figs. 10 and 11).

Peak areas of the derivatized relative to the underivatized PGs obtained from equal amounts of PGs are compared in Table III. PGE shows excellent response and selectivity, whereas the detection of PGF is impaired by less than half the response of

TABLE III

RELATIVE CHROMATOGRAPHIC RESPONSES (%) OF EQUAL AMOUNTS (100 ng) OF OXIDIZED AND NON-OXIDIZED PGs MEASURED AS: PEAK AREA (15-OXO-PG) (230 nm)/PEAK AREA (15-HYDROXY-PG) (190 nm)

| *PG* | *15-oxo-PG* | *By-products* |
|---|---|---|
| | *PG* | *PG* |
| $E_1$ | b(1): 140 | c(1): <1 |
| $E_2$ | b(2): 70 | c(2): <1 |
| $F_1$ | a(1): 58 | b(1): 6 |
| | | d(1): 12 |
| $F_2$ | a(2): 30 | b(2): 3 |
| | | d(2): 6 |

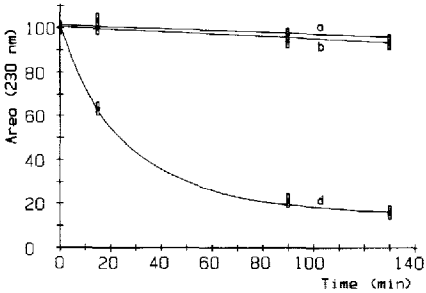



Fig. 13. Stability of oxidation products a, b and d from PGF in acetonitrile solution.

PGE and by-products at the 10–20% level. The response difference between oxidized $PG_1$ and $PG_2$ is not a property of the 15-oxo derivative, but reflects the larger absorptivity of underivatized $PG_2$ at 190 nm caused by the additional $\Delta^{5,6}$ double bond.

The low response of 15-oxo-PGF was, in addition to the effect of being distributed into several compounds, in part caused by the formation of compound d, which was not stable in solution, as shown in Fig. 13. Compounds a and b were stable for several hours in acetonitrile solution. This can be explained by the structural differences in the cyclopentane ring, since d possesses a reactive proton at C-12, being simultaneously allylic and α to a carbonyl group[47].

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