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CYCLIC DERIVATIVES FOR THE SELECTIVE CHROMATOGRAPHIC ANALYSIS OF BIFUNCTIONAL COMPOUNDS

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1. INTRODUCTION

This review is concerned with the chromatographic applications of chemically selective reagents forming cyclic derivatives with bifunctional compounds. The number of functionalized molecules in physiological fluids is immense and bifunctional compounds represent a small fraction of this physiologically important pool of substances. As such, they do not constitute a defined chemical class of substances like the steroids or terpenoids for example, but are found widely distributed among all classes of functionalized molecules (*e.g.* steroids, lipids, carbohydrates, nucleosides, catecholamines, prostaglandins, amino acids etc.). The distinctive physical feature that bifunctional compounds have in common is that they contain a minimum of two functional groups on a molecular framework which places these groups in close proximity to each other. No rigid geometrical rule can be given for the term "close proximity" as its meaning is related to the bridging ability of the reagent selected for the cyclization reaction. In general terms, bifunctional compounds can be considered to be those containing aliphatic chains with two functional groups on 1,2; 1,3; 1,4 carbon atom systems or aromatic compounds with *ortho* functional groups. The formation of larger rings than those represented by the above are rarely thermally or hydrolytically stable and are not suited for general chromatographic use.

As far as polar molecules are concerned, derivatization can be considered to be an auxillary technique in chromatography. Its impact on paper and thin-layer chromatography (TLC) has been small. Derivatives can be used to change relative mobilities of substances or to introduce a chromogenic tag to aid identification but neither technique is of necessity widely employed. In high-performance liquid chromatography (HPLC), most derivatization reactions are "detector orientated" and used to introduce UV-visible absorbing or fluorescent tags into substances with little or no native absorption in this region of the spectrum. This is a consequence of the current lack of a suitable universal and sensitive HPLC detector and the great reliance placed on the use of the UV-visible and fluorescence detector in routine analysis. It is probably in the gas chromatographic (GC) separation of polar molecules that derivatization has been most important. Initially derivatives were prepared to improve the thermal stability of polar molecules and to reduce their interaction with active centers in the chromatographic column and thus improve peak shape and separation characteristics. Derivatives have also been prepared to improve the resolution of complex mixtures by selectively removing a group of substances to a relatively empty region of the chromatogram and also as an aid to functional group identification through the characteristic changes in retention increments found after derivative formation with analysis on stationary phases of different polarity. Derivative formation is of considerable value in trace analysis as it provides a simple technique to introduce a tag into a molecule with a high response to a selective GC detector. This has considerably expanded the general usefulness and application of some detectors such as the electron-capture detector (ECD) into areas of research where it would otherwise have been of little value. The use of selective detectors and suitably designed derivatization reagents have a symbiotic relationship and both techniques have developed hand-in-glove with each other.

Early developments in derivatization were concerned with the search for universal one-step reagents which would react with all functional groups simultaneously.

The silylation and acylation reagents currently used today are successful examples of this research effort. Reagents of this type were designed to have the minimum of discriminatory power in chemical terms and consequently with complex mixtures they result in complex separation problems. In many analytical procedures, only a part of the sample is of interest and the rest (the matrix) is considered to be an interferent and must be separated either prior to analysis or by the resolving power of the chromatographic system. If the derivatization reaction could be made selective for the substance(s) of interest in the mixture and the reagent so designed that it could be used in conjunction with a selective detector for the determination, then the possibility exists for the development of simple analytical schemes for complex mixtures. Bifunctional compounds in mixtures could be selectively determined in this way.

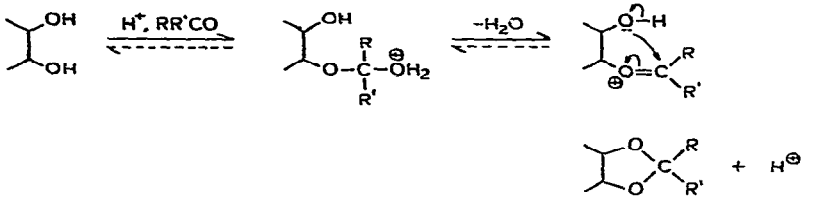
Information on selective reagents for the formation of cyclic derivatives has been recorded in general reviews of the techniques of derivatization[1–5]. Darbre has reviewed the use of cyclization reactions in chromatography[6].

2. ACETALS AND KETALS

Acetals and ketals are widely used in synthetic chemistry as protecting groups for *cis* diols and thiols. The derivatives are formed in high yield under mild conditions and are sufficiently stable to allow modification (*e.g.* esterification, oxidation, reduction etc.) to the underivatized portion of the molecule. The acetal and ketal protecting groups can be removed selectively by mild acid hydrolysis. The chemistry of the cyclic acetals of aldoses and aldosides[7,8] and of teritols, pentitols and hexitols[9] has been reviewed.

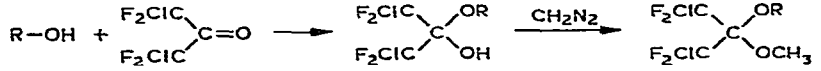
The impact of acetals and ketals in chromatography has been far less than their use in synthetic chemistry. Chromatography is often employed to monitor synthetic reactions involving acetals and ketals but this information is not specifically covered in this section unless the chromatographic step was important in the isolation or identification of the products.

Acetals and ketals are formed by the reaction between a ketone or aldehyde with a diol group on proximal carbon atoms. Generally, the ketone or aldehyde is used in excess, in many cases it may also be the solvent for the reaction, in the presence of an acid catalyst and/or a dehydrating reagent. Mechanistically, the reaction can be represented as follows:

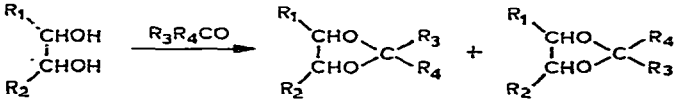


For aliphatic ketones and aldehydes, attack of the carbonium ion on the alcoholic oxygen atom results in the formation of a hemiketal which is rarely stable and rearranges to form the cyclic product. Substitution of the α-protons in the ketone or aldehyde with halogen atoms results in enhanced acidic character for the ketone group which confers much greater stability on the hemiketal intermediate and subse-

quent cyclization is no longer observed[10,11]. The hemiacetal formed between *syn*-dichlorotetrafluoroacetone or hexafluoroacetone and steroid hydroxyl groups could be methylated with diazomethane and then used for the determination of the steroid by GC[12].



The condensation of an unsymmetrical glycol and either an aldehyde or an unsymmetrical ketone can result in the formation of two stereoisomeric forms which differ only in respect of their configuration at the new asymmetric carbon atom (originally the carbon atom of the carbonyl group).

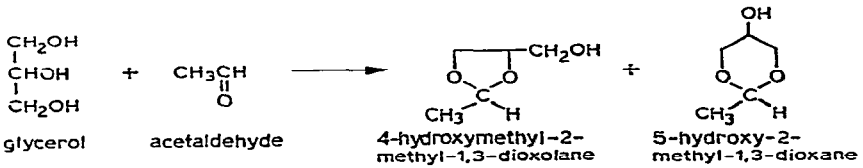


R_3, R_4 = H, alkyl or aryl group ($R_3 = R_4 = H$ exempted)

The product from the reaction between a diol and acetone is variously called an acetonide or an O-isopropylidene derivative. For uniformity, the term acetonide is adopted throughout this review.

2.1. Derivatives of lipids, glycerides and hydroxyacids

The uropygiols (2,3-*n*-alkane diols containing 22–24 carbon atoms) of structure $CH_3(CH_2)_{18-20}CHOHCHOHCH_3$ were extracted from chicken preen glands and identified in part by GC–mass spectrometry (MS) of the acetonide derivatives[13]. The three uropygiol acetonide derivatives were separated into six peaks on GC consisting approximately of equal amounts of the *threo* and *erythro* isomers. The prominent $[M-15]^+$ ion in the mass spectra of the acetonide derivatives served to indicate the molecular weight of the original diol. The methyl ester of veronica oil (mainly *cis*-12, 13-epoxy-*cis*-O-octadecanoate) forms a diacetonide derivative separated into the two diastereoisomeric products on GC[14]. The double bond was epoxidized and then both epoxide groups converted to the acetonide with acetone and boron trifluoride–etherate catalyst. Wood[15] has investigated the use of GC and TLC for the separation of unsaturated fatty acids by permanganate oxidation of the double bonds, methyl ester formation of the carboxylic acid group and acetonide formation of the double bond diols. The complex mixtures of *threo* and *erythro* isomers formed by fatty acids with multiple double bonds could only be partially resolved by GC and TLC. The acetonide derivatives of monounsaturated fatty acid esters were easily separated by GC. Positional acetonide derivatives, derived from positional isomers of monounsaturated fatty acids were not separated by GC but could be resolved by TLC.



Acetaldehyde and acetone react differently with glycerol[16]. Acetaldehyde

produces a mixture of four products corresponding to the *cis* and *trans* isomers of 4-hydroxymethyl-2-methyl-1,3-dioxolane and 5-hydroxy-2-methyl-1,3-dioxane which are separated by GC. Benzaldehyde has been shown to react in a similar manner with glycerol[17]. The reaction between glycerol and acetone produces only the dioxolane product. Acetonide derivatives have been prepared from α-monoglycerides (β-isomers do not react) esterified with C_2–C_{18} fatty acids and separated by GC[18]. The acetonide derivatives of α-monoglycerides esterified with unsaturated fatty acids are eluted after their saturated homologues on the selective ethylene glycol succinate stationary phase[19]. These derivatives were stable to TLC and column chromatography using silicic acid. Ketal derivatives formed with acetone, methyl ethyl ketone, methyl isobutyl ketone, heptan-2-one, cyclopentanone and cyclohexanone have been used for the GC separation of mono and diepoxyglycerides[20]. The epoxides were converted to the ketals by reaction of the appropriate ketone in the presence of boron trifluoride–etherate at room temperature for 2 h (*cf.* ref. 14). Under these conditions, conversion to the ketal was not complete; for example trivernolin gave 31 % of the 1,3-dioxolane derivative with cyclopentanone. For the analysis of natural products, cyclopentanone ketals were recommended as the epoxyglyceride derivatives were separated from the co-extracted C_{36} to C_{66} triglycerides.

The 2-hydroxy fatty acids from lipid hydrolyzates were determined by GC–MS after acetonide formation[21]. The 3-hydroxy fatty acids do not form acetonides and are easily differentiated from their 2-hydroxy analogues.

2.2. Derivatives of carbohydrates and nucleosides

Acetonide derivatives of fructose and glucose could be separated by paper chromatography[22]. The derivatives were formed using an ion-exchange catalyst but the reaction was unfortunately slow and did not always proceed to completion. In a more extensive study of the separation of carbohydrate acetonides by paper chromatography, good separations were obtained by reversed-phase ascending chromatography on cellulose acetate strips at 2–5° using methanol–water (6:4, v/v) as eluting solvent[23]. Carbohydrates with different numbers of unreacted hydroxyl groups have characteristic mobility values (Table 1) which aids their identification. TLC has been used to monitor carbohydrate reactions and a few R_F values for carbohydrate acetonide derivatives given[24].

TABLE 1

REVERSED-PHASE PAPER CHROMATOGRAPHY OF SOME CARBOHYDRATE ACETONIDE DERIVATIVES

Derivative	R_F	*Number of free hydroxyl groups*
1,2:4,5-Diacetonide-3-mesyl-D-fructose	0.15	0
DL-Methylglycerate acetonide	0.28	0
1,2:3,4-Diacetonide-6-tosyl-D-galactose	0.32	0
Methyl-2,3-acetonide-αβ-D-ribofuranoside	0.48	1
1,2:5,6-Diacetonide-D-glucose	0.52	1
1,2:3,4-Diacetonide-D-galactose	0.55	1
1,2-Acetonide of glycerol	0.65	1
1,2-Acetonide-D-glucofuranose	0.80	3
3-O-Methyl-D-glucose	0.98	4

Hedgley *et al.*[25] were the first to use GC for the separation of carbohydrate acetonides. The authors described the separation on a preparative scale of the monoacetonides of 5- and 6-deoxyglucose as very satisfactory and indicated that the method was applicable to the acetonide derivatives of hexoses and pentoses. Jones *et al.*[26] described the separation by GC of many acetonide, benzylidene, ethylidene and carbonate derivatives of carbohydrates. The derivatives were separated with free hydroxyl groups or after acetylation which reduced retention volumes. The separation of acetonide derivatives of carbohydrates has also been discussed by Kircher[27]. Arzoumanian *et al.*[28] analyzed the 2,3-acetonide derivatives of methyl 5,6-dideoxy-β-D-allofuranoside-4-ene, methyl 5,6-dideoxy-β-D-glucofuranoside, methyl 6-deoxy-β-D-allofuranoside and methyl 6-deoxy-β-D-gulofuranoside by GC. Sweeley *et al.*[29] noted that acetals formed from anomeric sugar mixtures gave multiple peaks on GC and also that the benzylidene derivative of α and β-idosides was unsuitable for their resolution on a packed column of SE-52. It was shown that acetonide derivatives of carbohydrates with free hydroxyl groups may undergo rearrangement on GC unless the free hydroxyl groups are also protected by formation of acyl ester derivatives[17]. A brief report has appeared on the use of acetonide derivatives to determine monosaccharides in spent sulfite liquors[30]. However, the method employed for the formation of the acetonide derivative was lengthy and unsuitable for routine use.

Butyraldehyde catalyzed by hydrobromic or hydrochloric acid reacts with derivatives of D-glucitol (1-dexoy-D-glucitol, 2-deoxy-D-glucitol and 3-O-methyl-D-glucitol) to give mixtures of products depending on the experimental conditions employed which could be separated by GC[31]. Benzylidene derivatives of monosaccharides have been prepared for the characterization of sugar *endo-* and *exo*-stereoisomers[32]. The benzylidene derivatives containing a free hydroxyl group gave a good separation of the isomers on GC; the *endo* isomers having shorter retention times.

Syrupy mixtures of aldoses (fucose, arabinose, xylose, rhamnose, galactose, glucose, mannose) could be converted to their acetonide derivatives at room temperature by a 2-h reaction with acetone containing 1% sulfuric acid as catalyst and the derivatives completely separated by GC (Table 2)[33]. For glucose and rhamnose, a small peak, in addition to the main peak was observed and glucose also gave traces of a third product.

The retention times of 2′,3′-acetonide derivatives of uridine and adenosine have been reported[34]. The electron-impact mass spectrum of uridine, adenosine, cytidine and guanosine as their acetonides, acetonide trimethylsilyl ether, acetonide acetate and acetonide trifluoroacetate derivatives have been recorded[35]. The acetonide group showed a characteristic elimination of a methyl group and acetone from the molecular ion.

The acetonide derivatives of carbohydrates are structural isomers as opposed to the stereoisomers formed with monodentate reagents and as such show strong characteristic and diagnostic modes of fragmentation in MS. Many mass spectra of the acetonide derivatives have been recorded and only the most salient features of these spectra will be dealt with here. For a more complete treatment, specialist reviews on the mass spectra of carbohydrates should be consulted[8,36–38].

The electron-impact mass spectra of carbohydrate acetonides have weak or absent molecular ions. A fragment ion at $[M-15]^+$ due to loss of a methyl group from the acetonide ring serves to identify the molecular weight of the original sugar.

TABLE 2

CHROMATOGRAPHIC DATA FOR GC OF ALDOSE ACETONIDE DERIVATIVES

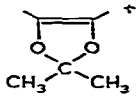
Aldose	*Main acetonide*	*Relative retention time**	
		*OV-225***	*XE-60****
L-Fucose	1,2:3,4(α)	0.20	0.32
L-Arabinose	1,2:3,4(β)	0.24	0.38
D-Xylose	1,2:3,5(α)	0.39	0.49
L-Rhamnose	2,3	0.72	0.82, (0.78)
D-Galactose	1,2:3,4(α)	0.86	0.90
D-Glucose	1,2:5,6(α)	0.90 (0.82, 0.59)	0.94 (0.87, 0.60)
D-Mannose	2,3:5,6	1.00	1.00

* 2,3:5,6-Diacetonide of D-mannose used as internal standard.

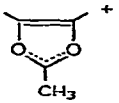
** 2 m × 2 mm I.D. column of 3% OV-225 on Gas-Chrom Q (80–100 mesh), nitrogen flow-rate 20 ml min^{-1}. Temperature programme: isothermal, hold at 110° for 12 min, then 1.5° min^{-1}. Retention time of internal standard approximately 39.0 min.

*** 6 ft. × 1.5 mm I.D. column of 3% XE-60 on Chromosorb G (80–100 mesh), nitrogen flow-rate 20 ml min^{-1}. Temperature programme: isothermal, hold at 120° for 5.0 min, then 2° min^{-1}. Retention time of internal standard approximately 34.0 min.

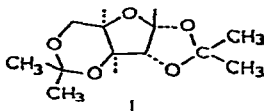
Further fragmentation of this ion by sequential elimination of the elements of acetone and acetic acid enable the number of acetonide groups in the molecule to be identified. Prominent ions of *m/e* 43 (C_2H_3O) and *m/e* 59 (C_3H_7O, protonated acetone) are characteristic of the acetonide ring. Diacetonide derivatives have characteristic ions at *m/e* 100 and *m/e* 85 while the monoacetonide derivatives usually only have the ion of *m/e* 85. The diacetonide derivatives of pentoses and hexoses undergo characteristic fragmentation ("h-rupture") to produce ions of *m/e* 101, 129 and 159.

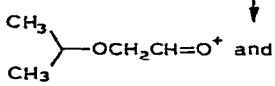


m/e 100

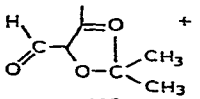


m/e 85



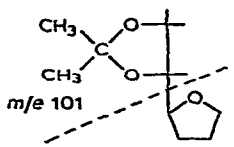


m/e 101



m/e 129

The acetonide derivatives can be used to distinguish between the furanose and pyranose isomers of sugars by mass spectrometry. The furanose acetonide derivatives

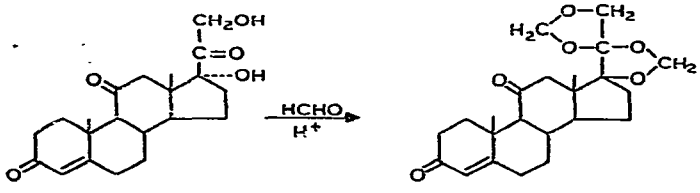


m/e 101

show a characteristic fragmentation involving rupture of the C-4, C-5 bond to produce a stable ion at *m/e* 101[39–42]. This ion is only of diagnostic importance when relatively abundant.

2.3. Derivatives of steroids

The C-17 side chain of the corticosteroids is thermally labile and for the GC analysis of these compounds appropriate derivatives have to be prepared. Cortisone reacts slowly with formalin in acid media to form bismethylenedioxy derivatives[43].



Cortisone Bismethylenedioxy derivative

As well as cortisone a series of 17-hydroxycorticosteroids were investigated and in nearly all cases one major peak was obtained on GC attended by shoulders or small secondary peaks. The results obtained with corticosteroids containing ring hydroxyl groups were unsatisfactory even after the formation of acetate derivatives. Corticosteroids with *cis*-C-20,21- or -C-17,20-dihydroxyl groups form acetonide derivatives under mild conditions[44–46]. The acetonide derivatives are suitable for separation by TLC and GC. The acetonide group was also stable to the conditions necessary to form trimethylsilyl (TMS) ethers, acetates or methoximes of other functional groups present in the molecule. The reaction was specific for *cis*-diols as the *trans*-C-20,21-diol did not form an acetonide derivative. Estetrol (1,3,5(10)-estratriene-3,15α,16α,17β-tetrol) forms a 15α,16α-acetonide derivative which is stable to aqueous acid and base and to the conditions necessary for the formation of the TMS ethers of the remaining hydroxyl groups[47,48]. Trimethylchlorosilane was used as catalyst for acetonide formation. The epimers of estriols with *cis*-C-16,17-dihydroxyl groups are well separated as their acetonide derivatives on TLC using silica gel layers modified with ammonium bisulfite[49]. The *trans* epimers do not form acetonide derivatives.

Mono- and diacetonide derivatives have been employed for the TLC separation and identification of the insect moulting hormones, ecdysones[50,51]. The mass spectra of these derivatives have been recorded[50,52]. Acetonide derivatives were used to confirm the presence of 3-epiecdysterone in the meconium fluid of the tobacco

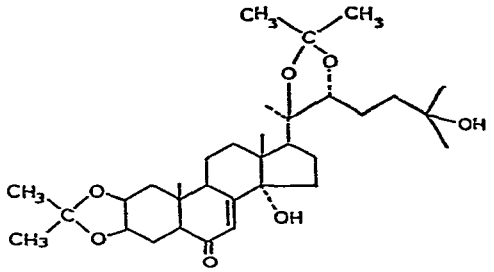
TABLE 3

RELATIVE RETENTION TIMES OF 2β,3β-DIHYDROXY-5α-CHOLESTANE DERIVATIVES

3 ft. column of 1% OV-101 on Gas-Chrom Q, column temperature 260° and nitrogen flow-rate 80 ml min^{-1}.

Compound	*Retention time (min)*
2β,3β-Dihydroxy-5α-cholestane	2.40
Acetonide derivative	2.10
Di-TMS derivative	2.70
n-Butaneboronate derivative	3.60

hornworm[53]. The *trans*-2β,3α-diol in this hormone does not form an acetonide derivative and this was used with other evidence to distinguish this hormone from ecdysterone. A convenient micro method has been developed for the formation of acetonide derivatives of ecdysone analogues[54]. The steroid (0.2 mg) was dissolved in acetone (500 μl) and phosphomolybdic acid added as catalyst (6 μl of a solution containing 1 μg/μl in acetone). The reaction was complete in 30 min at room temperature. The acetonide derivatives were more volatile than the TMS ethers or *n*-butaneboronates (Table 3). The acetonide derivatives were cleaved by trimethylsilylimidazole under mild conditions.

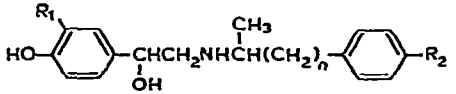


2,3.20,22-diacetonide derivative of ecdysterone

2.4. Derivatives of catecholamines and amino alcohols

Ephedrine and related catecholamines with at least one unsubstituted proton on nitrogen can be converted to acetonide derivatives which are stable to GC[55–57]. In one case, silica gel was used as catalyst and the reaction was complete in 6 h at reflux in acetone[57]. The diastereoisomers of *l*-ephedrine and *d*-pseudoephedrine were reported as sharp peaks on a PEG 6000 column. Norephedrine reacts with acetone to form the Schiff base and methylephedrine does not react under the conditions employed for acetonide formation. Salmefanol and labetol react with acetone, benzaldehyde and anisaldehyde to form cyclic derivatives which were found to be unstable towards GC[58].

Ephedrine

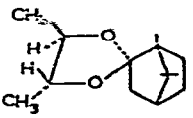


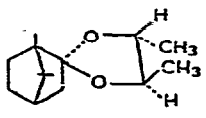
Salmefa $R_1 = CH_2OH$, $R_2 = OCH_3$, $n = 1$

Labetalolnol $R_1 = CONH_2$, $R_2 = H$, $n = 2$

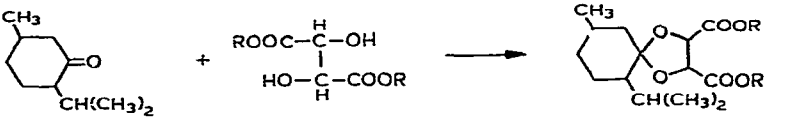
2.5. Derivatives for enantiomer separation

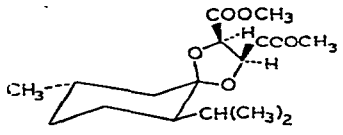
$\pm$ Camphor reacts with D-(—)-2,3-butanediol in the presence of *p*-toluenesulfonic acid to give the two possible diastereoisomers which were separated by GC on both an analytical and preparative scale[59]. Glass capillary columns were used to re-

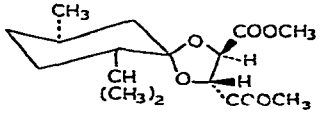

Diastereoisomer


Derivatives of Camphor

solve the diastereoisomeric ketals formed between (+)-dimethyl tartrate or (+)-2,3-butanediol and *sec.*-butylmethylketone, 2-methylcyclohexanone, 3-methylcyclohexanone, 2,4,4-trimethylcyclopentanone and 3,3,5-trimethylcyclohexanone[60]. The diastereoisomers formed between (±)-menthone and (+)-tartaric diesters (dimethyl, diethyl, diisopropyl and dibutyl) were separated by GC using packed columns of Carbowax 20 M[61]. The resolution obtained with 2,3-butanediol was poor and the use of (+)-dimethyl tartrate was found to give the best separation. The (+)(+)-diastereoisomers always had longer retention times than the (−)(+)-diastereoisomers for the tartaric diester reagents used.

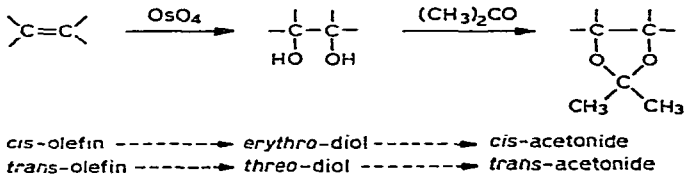

Menthone + (+)-Tartaric acid Diester → Diastereoisomeric ketal


(−)(+)-Diastereoisomer


(+)(+)-Diastereoisomer

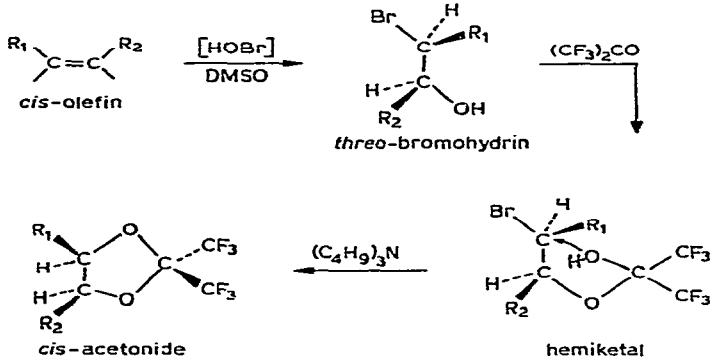
2.6. Derivatives for double bond location in alkenes

The location of the position of a double bond in a long-chain alkene by MS is difficult. Under the conditions prevailing in the mass spectrometer, the ionized molecular ion undergoes facile isomerization prior to fragmentation with a scrambling of the information concerning the location of the double bond and its geometrical configuration. The net result is a series of near identical mass spectra from which structural interpretation is impossible. A solution to this problem is the formation of derivatives of the double bond in a stereospecific way which yield fragments characteristic of the location of the double bond and intensity differences characteristic of the geometric arrangement of substituents attached to it.

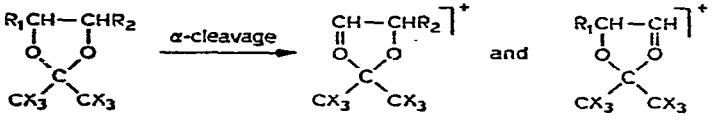

cis-olefin ⟶ *erythro*-diol ⟶ *cis*-acetonide
trans-olefin ⟶ *threo*-diol ⟶ *trans*-acetonide

The double bond can be conveniently oxidized in olefins[62] and unsaturated fatty esters[63,54], stereospecifically to the *cis*-diol, with osmium tetroxide. The diol can then

be converted to the acetonide derivative or cyclic boronate (see section on boronic acids) without changing the original configurational identity of the double bond. The use of $Os^{18}O_4$ and d^6-acetone enables heavy isotope labeling to be conveniently carried out as an aid to mass spectral interpretation. Hexafluoroacetone does not condense with diols and acetonide derivatives with this reagent are formed via the bromohydrin[11]. The bromohydrin adduct is *trans* specific (at least 97%) and formation of the hexafluoroacetone ketal involves base-promoted backside attack on the bromine containing carbon atom with inversion of the configuration. A disadvantage of this reaction is the long time (2–3 days) required for derivatization and the moderate overall yield (85%). The advantages are that the derivatives are volatile with good GC properties and the presence of fluorine enables fragmentation pathways in the mass spectra involving the acetonide ring to be easily distinguished from those occurring in the hydrocarbon portion.

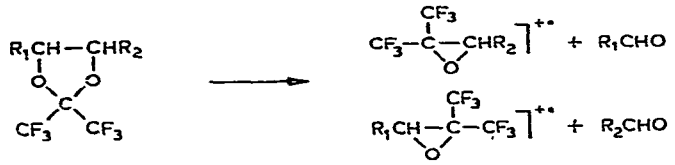


The mass spectra of all derivatives are characterized by a weak or absent molecular ion and a prominent $[M-CH_3]^+$ ion ($M-CF_3$ in the case of the hexafluoroacetone derivative) which serves to indicate the molecular weight of the original alkene. The tertiary carbonium ion formed is well stabilized by the two ether oxygen atoms and this constitutes a localized charged center from which the principal modes of fragmentation are initiated. Further loss of ketene (*m/e* 42) results in the formation of a protonated expoxide ion (not observed with hexafluoroacetone acetonides) and also loss of acetic acid in the case of the fatty acid esters (not observed with alkenes). In the lower mass range, two of the most prominent ions observed in all spectra *m/e* 43 (acetyl ion) and *m/e* 59 (protonated acetone) are formed from the acetonide ring (not always prominent as their fluorinated analogues in hexafluoroacetonides).



The position of the double bond in the molecule is indicated by simple α-cleavage to form two ions containing either of the end groups of the original double bond. For the hexafluoroacetonide derivatives the presence of ions 29 a.m.u. below the fragments formed by α-cleavage permits an unequivocal assignment of the double

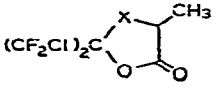
bond in the original alkene to be made. In the mass spectra of acetonides, secondary ions are formed from the fragments of α-cleavage as well as mass discrimination effects (substituents with the bulkiest R group tend to be more intense). For dienoic and trienoic unsaturated fatty acid acetonides, the number of double bonds can be established from the consecutive losses of acetone (*m/e* 58) from the $[M-15]^+$ ion. The position of the double bonds is less obvious as only those ions containing solely one acetonide ring are observed by α-cleavage[64]. An interpretation can be made from the lower mass hydrocarbon fragments but this is less convenient and more prone to error.



As might be expected, the *erythro* and *threo* isomers of monoalkenes give rise to mass spectra which show intensity but not mass differences. However, unless suitable standards are available for comparison and the information is coupled with retention index values obtained by GC, the assignment of geometrical configuration is difficult[11,63,64]. As a general observation, the acetonide formed from a *cis* double bond produces more prominent ions by α-cleavage than does the trans isomer[11,62–64]. Intensity differences are usually in the range of two- to four-fold.

3. OXAZOLIDINONES

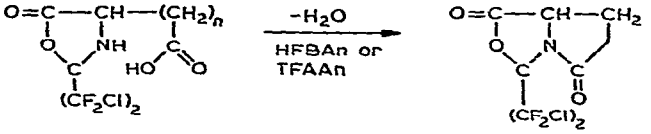
Substitution of hydrogen with electronegative groups such as chlorine or fluorine in acetone enhances the acidic character of the carbonyl group and promotes the formation of a series of stable monofunctional adducts and cyclic derivatives not observed with aliphatic ketones[10]. The condensation of hexafluoroacetone[65] and 1,3-dichlorotetrafluoroacetone[65,66] with α-substituted carboxylic acids leads to the formation of a series of stable five-membered ring derivatives.



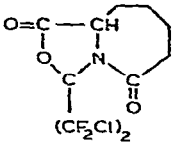
X = NH 1,3-oxazolidin-5-one
X = O 1,3-dioxolan-5-one
X = S 1,3-oxathiolan-5-one
X = CO also known

Some of these derivatives were found to be suitable for gas chromatography after silylation of protonic groups in the side chain with hexamethyldisilazane[65–67]. For the preparation of amino acid derivatives (X = NH), the conditions employed required elevated temperatures, long reaction times and polar solvents. The amino acid hydrochloride salts could not be derivatized under these conditions. Hušek investigated the reaction conditions for the formation of the oxazolidinones of tyrosine and its 3-iodo and 3,5-diiodo analogues[68,79]. The solubilizing power of the solvent was found to be important and the addition of a trace of pyridine (1 % in acetonitrile) rapidly dissolved the amino acid, its ammonium salt or hydrochloride salt enabling the condensation reaction with 1,3-dichlorotetrafluoroacetone to proceed smoothly and rapidly at room temperature. The use of strong bases such as triethylamine or N-methylpyrroli-

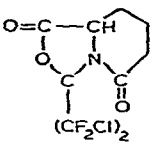
dine in place of pyridine resulted in a loss of derivative. Silylation of the phenolic group of tyrosine with N,O-bis(trimethylsilyl) acetamide gave unsatisfactory results. The preferred method was acylation of the phenolic group with trifluoroacetic anhydride or heptafluorobutyric anhydride which was carried out by addition of the anhydride to the condensation medium without removal of excess reagent. The derivatives so formed were very sensitive to the ECD and could be determined at the picogram level[70]. With dicarboxylic amino acids such as aspartic acid, glutamic acid, aminoadipic acid and 2-aminopimelic acid, the use of heptafluorobutyric anhydride to derivatize the unreacted polar groups of the oxazolidinones gave unsatisfactory results[71]. No peak was observed for aspartic acid and multiple peaks were observed for the other dicarboxylic amino acids. This was explained by the possibility of the formation of bicyclic derivatives under the influence of the strong dehydrating action of the acid anhydride.



bicyclic derivative of glutamic acid

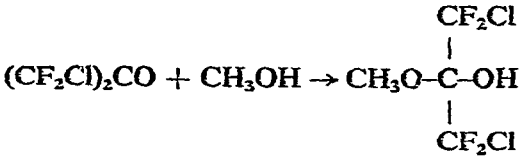


bicyclic derivative of aminopimelic acid



bicyclic derivative of aminoadipic acid

Derivatization of the second carboxylic acid group was carried out without significant by-product formation by addition of an alcohol to the condensation medium. For example, the addition of methanol immediately gave the expected hemiacetal, which becomes the reagent for the esterification reaction.

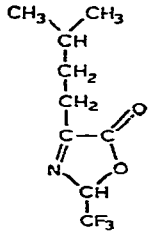


1,3-dichloro-1,1,3,3-tetrafluoro-2-methoxypropan-2-ol

Further protonic groups in the side chain can be acylated with heptafluorobutyric anhydride after extraction and purification of the oxazolidine ester to give derivatives suitable for GC. As the condensation reaction occurs under mild conditions in weakly basic media, attack of the amino acid amide group was not observed making the method suitable for the simultaneous analysis of aspargine and glutamine in admixture with aspartic and glutamic acids[72].

Amino acids (α-aminobutyric acid, α-amino-α-methylbutyric acid, alanine,

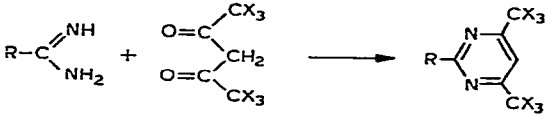
leucine, alloisoleucine, valine, cycloleucine, phenylglycine and phenylalanine) upon heating in a sealed tube with trifluoroacetic anhydride (150° for 10 min) have been shown to form inner esters with a 4-substituted-2-trifluoromethyloxazoline-5-one structure[73,74]. The 2-trifluoromethyloxazolin-5-ones were more volatile than the N(O)-trifluoroacetyl-O-butyl ester derivatives and were well separated on GC. This reaction has been applied to the measurement of phenylalanine in serum[75]. None of the amino acids investigated had protonic groups in the side chain.



Leucine oxazolinone derivative

4. CYCLIC DERIVATIVES OF BIGUANIDES

Biguanides of pharmaceutical importance can be converted to cyclic derivatives suitable for GC by condensation with acetylacetone[76–79] or hexafluoroacetylacetone[79–81] to form substituted pyrimidines. The structures of the biguanides studied are summarized in Table 4.

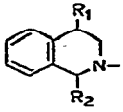
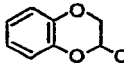
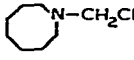
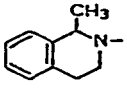
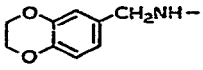


X = H or F

Acetylacetone can be made to react directly with biguanides in aqueous physiological fluids at slightly alkaline pH (sodium bicarbonate buffer)[78,79]. The reaction is incomplete and for quantitative analysis a related substance is required as an internal standard[77,79,82]. An alternative method (used with hexafluoroacetylacetone) employs two phases using benzene or toluene as the extraction solvent for the derivative. An advantage of this method is that very few interfering substances are extracted from blood or urine. For studies on the metabolism of biguanides in physiological fluids, very sensitive techniques are required and selective GC detectors are frequently used. Debrisoquine was determined with the nitrogen–phosphorus detector (NPD) in urine, plasma and saliva with a minimum measurable concentration of 3.0 ng ml^{-1} (ref. 79). Using mass fragmentography and tetradeuterio-debrisoquine as an internal standard, the least detectable amount of debrisoquine and its 4-hydroxy metabolite were 1.0 ng ml^{-1} and 5.0 ng ml^{-1} in plasma respectively[78,81]. The detection limit of the hexafluoroacetylacetone derivative of guanbenzodioxan was 25 ng ml^{-1} with the ECD and approximately one order of magnitude lower when determined by mass fragmentography in biological fluids[82]. The minimum detectable concentration of debrisoquine hexafluoroacetylacetone derivative was $5 \cdot 10^{-13}$ g of drug on column (corresponding to 25 ng ml^{-1} in plasma) with the ECD. Modification

TABEL 4

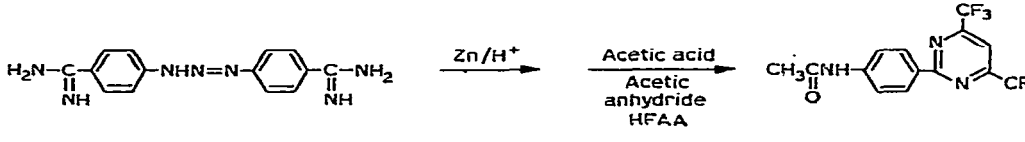
BIGUANIDES OF STRUCTURE RC(NH)NH$_2$ FORMING SUBSTITUTED PYRIMIDINE DERIVATIVES

R Group	*Name*	*Reagent*	*Ref.*
$-NH_2$	Guanidine	Acetylacetone	76, 83
CH_3 NH–	Methylguanidine	Acetylacetone	76, 83
$(CH_3)_2$ N–	Dimethylguanidine	Acetylacetone	76
	R_1=R_2=H, Debrisoquine R_1=OH, R_2=H, 4-Hydroxydebrisoquine	Acetylacetone Hexafluoroacetyiacetone	77 79 81
	Guanoxan Guanethidine	Acetylacetone Hexafluoroacetylacetone	79 80, 82
	3,4-Dihydro-1-methyl 2(1*H*)-isoquinolinecarboxamide	Acetylacetone	79
	Guabenzoidioxan	Hexafluoroacetylacetone	82
			

of the extraction procedure should enable much lower concentrations in biological fluids to be determined[81].

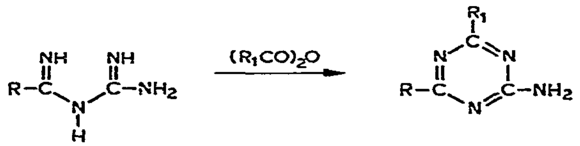
Seven derivatives (hexafluoroacetylacetone (HFAA), trifluoroacetylacetone, acetylacetone, trifluoroacetyl, acetyl, silyl, isothiocyanate) were evaluated for the GC analysis of guanidine, methylguanidine and agmatine[83]. The cyclic derivatives had the most favorable properties of which the hexafluoroacetylacetonate derivatives were recommended as these were the most volatile and hydrolytically stable. For derivatization a sealed tube reaction at 120° for 1 h with pyridine–HFAA (1:1) gave essentially complete reaction (100% for guanidine, methylguanidine and 97.4% for agmatine). With the AFID the least detectable amount of guanidine and methylguanidine was 5.0 ng and 15.0 ng for agmatine. With the ECD detection limits were 50 pg for guanidine, methylguanidine and 150 pg for agmatine.

Diminazene has been determined as the acetyl hexafluoropyrimidine derivative of 4-aminobenzamidine in plasma after a double acid reduction treatment in which the pyrimidine derivative is formed in the second stage in acid solution[84].



Diminazene could not be extracted from aqueous solution even at high pH and a double reduction technique in acid solution was necessary to ensure complete conversion to 4-aminobenzamidine otherwise unsatisfactory results were obtained. By chemical ionization MS using methane as the reagent gas and monitoring the ion $[MH]^+$, diminazene levels in the range 0.1 to 10 μg ml^{-1} of plasma were determined.

The reaction between biguanides and organic acid anhydrides results in the formation of cyclic 2,4-disubstituted-2,6-amino-1,3,5-*s*-triazines which have good thermal and GC properties[85–87]. The structures of the biguanides studied are summarized in Table 5. The reaction of eight biguanides by either heating with anhydride or in a sealed tube reaction with anhydride and triethylamine to give yields of between 50–90% of the *s*-triazine have been studied and the products characterized by elemental



analysis and physical spectroscopy (UV, NMR, MS)[86]. The acid anhydrides tested include chlorofluoroacetic, dichlorofluoroacetic, heptafluorobutyric, pentafluoropropionic and trifluoroacetic anhydride. For the analysis of biological fluids, the drugs are first extracted from plasma after protein precipitation and basification into an organic solvent for derivative formation. Using chlorodifluoroacetic anhydride as cyclizing reagent, phenformin could be determined with an ECD at below the 1.0 ng ml^{-1} level in plasma[85]. Using trifluoroacetic anhydride as cyclizing reagent and chemical ionization mass fragmentography 1–5.0 ng ml^{-1} of phenoformin were determined in plasma[87]. Under electron-impact conditions, most of the *s*-triazines fragment by α-cleavage to give the base peak of the mass spectrum and it has been suggested that this would be suitable for the analysis of physiological levels by mass fragmentography[86].

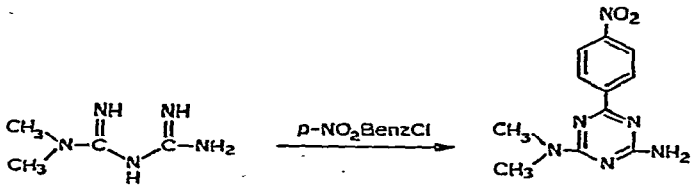
Metformin has also been determined by HPLC after formation of the UV-

TABLE 5

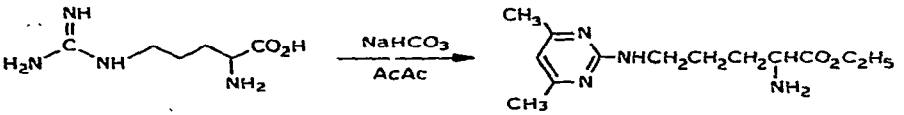
BIGUANIDES OF STRUCTURE $RC(NH)NHC(NH)NH_2$ FORMING CYCLIC SUBSTITUTED *s*-TRIAZINE DERIVATIVES

R	*Name*	*Ref.*
$C_6H_5CH_2CH_2NH-$	Phenformin	85, 87
C_4H_9NH-	Buformin	85, 86
$(CH_3)_2N-$	Metformin	86, 88
C_3H_7NH-	1-Propylbiguanide	86
$C_6H_5CH_2NH-$	Benzylbiguanide	86
$CH_3C_6H_4CH_2CH_2NH-$	1-(*p*-Methylphenylethyl) biguanide	87
$CH_3OC_6H_4CH_2CH_2NH-$	1-(*p*-Methoxyphenylethyl) biguanide	87
$C_6H_5CH_2CH(CH_3)NH-$	*d*-1-(α-Methylphenylethyl) biguanide	87
$C_5H_{11}NH-$	1-Pentylbiguanide	87
$C_6H_5CH_2CH_2C_2H_5N-$	Amformin	87

sensitive *p*-nitrobenzoyl derivative by addition of *p*-nitrobenzoyl chloride to urine made alkaline with sodium hydroxide[88].

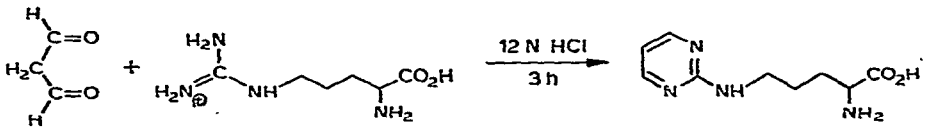


Concentrations of methformin as low as 0.02 mg 100 ml^{-1} of urine were determined in this way. Methylguanidine, guanidine and other guanidino compounds react with 9, 10-phenanthrenequinone to form a highly fluorescent product which can be detected at the low nanomole level (0.49 ng for guanidine, 0.63 ng for methylguanidine) with a fluorescence detector after separation of the guanidino compounds by cation-exchange HPLC[89,90]. The derivatives were formed by a post-column reaction in a stainless steel delay coil (1 min at 75°). The method was suitable for determining guanido compounds in as little as 1.0 ml of serum or 0.2 ml of cerebrospinal fluid obtained from uremic patients.



Arginine Ethyl ester

The guanidino function of the amino acid arginine reacts with acetylacetone to form a pyrimidine derivative which after esterification of the carboxylic acid function is suitable for the analysis of arginine by paper chromatography and GC[91]. MS can be used to determine the sequence of all common amino acids in polypeptides except for arginine which exhibits no specific fragmentation behavior. Conversion of arginine to its pyrimidine derivative provides cyclic derivatives suitable for the amino acid sequencing of polypeptides containing arginine[92]. Arginine reacts with malonaldehyde (yields 83–100%) to form S–N–(2-pyrimidinyl)–L–ornithine.

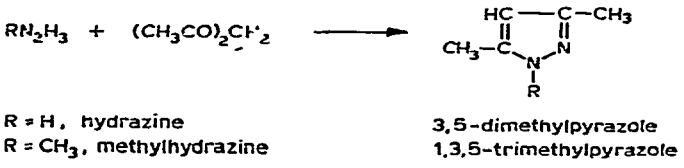


Benzoyl-L-arginine and malonaldehyde in ethanol as solvent produced simultaneous condensation of the guanidino group and formation of the ethyl ester without racemization. The derivative formed was volatile and should be suitable for GC and MS studies of polypeptides containing arginine residues[93]. GC–MS was used to determine the trifluoroacetylated dimethylpyrimidyl derivatives of guanidinoacetic acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, arginine and homoarginine in rat and bovine brain[94]. The dimethylpyrimidyl derivatives of the guanidino group were prepared in pyridine–water (1:1) at pH 8.0–9.0 (adjusted by addition of sodium bicarbonate) by the addition of acetylacetone and refluxing at 100° for 10 h. For chro-

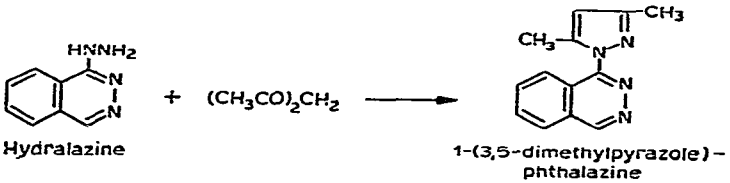
matography, the carboxylic acid groups were protected by conversion to their *n*-butyl esters and free amino groups were trifluoroacetylated. By mass fragmentography a detection limit of less than 1.0 ng could be obtained for these compounds.

5. CYCLIC DERIVATIVES OF HYDRAZINES

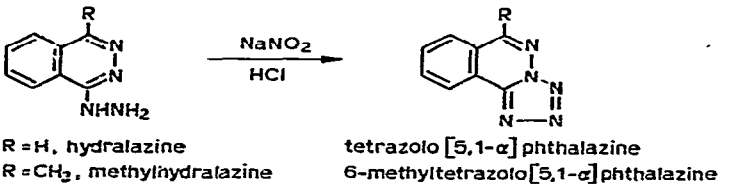
Hydrazine and methylhydrazine react with 2,4-pentanedione in aqueous solution of pH 6–9 to form cyclic pyrazoles. The reaction was complete within 1 h at room temperature and aliquots of the aqueous solution were injected directly into the



GC for the determination of hydrazine in the range 0.1 to 50 ppm[95]. At 100-fold excess concentration, Fe^{3+} and Cu^{2+} interfered markedly in the determination unless masked by the addition of Na_2EDTA and adjusting the pH of the solution to 5.5 before adding the 2,4-pentanedione. Hydralazine in tablet form was determined by GC after formation of its phthalazine derivative in aqueous solution with 2,4-pentanedione[96]. The derivative was extracted into an organic solvent for GC analysis and the



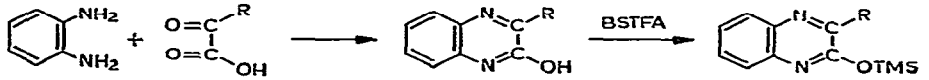
injection port temperature was maintained low (210°) to avoid the possibility of thermal decomposition. Stationary phase (SE-30) loadings greater than 6% (w/w) on Gas-Chrom Q were required to give reproducible response factors for the derivative using phenanthrene as internal standard. At acid pH, hydralazine reacts with sodium nitrite in aqueous solution to form a tetrazolophthalazine derivative[97]. The yield of the derivative was 85–95% so that for quantitative analysis methylhydralazine was employed as internal standard. Extraction of the derivative by an organic solvent at pH 10 and analysis by GC–ECD enabled as little as 10 ng ml^{-1} of hydralazine to be



determined in plasma. A similar reaction with nitrous acid was used to determine dihydralazine in plasma with methylhydralazine as internal standard by reversed-phase HPLC[98]. The limit of detection for dihydralazine at $\lambda = 230$ nm was 2.5 ng ml^{-1} of plasma. It has been suggested that the acidic conditions used in the derivative reaction could cause hydrolysis of acid-labile conjugate metabolites resulting in an elevated level for the free dihydralazine concentration[99]. Lowering the pH of the solution to 3.0 for formation of the derivative should enable the real dihydralazine concentrations to be determined.

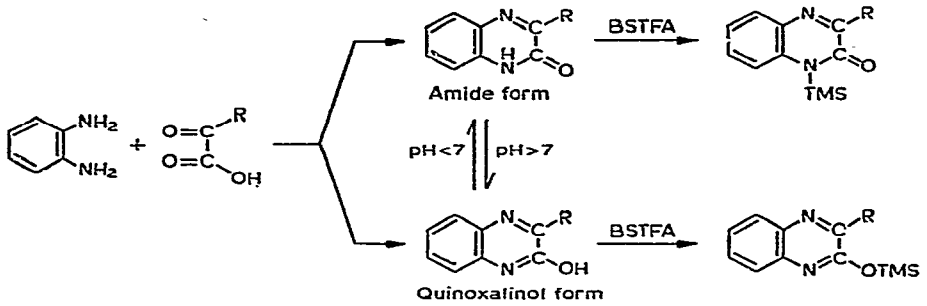
6. QUINOXALINOL DERIVATIVES OF α-KETO ACIDS

1,2-Diaminobenzene selectively reacts with α-keto acids in acid solution to form quinoxalinols which are thermally and hydrolytically stable cyclic derivatives.

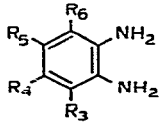


For gas chromatography, the derivatives are converted to their trimethylsilyl ethers. Optimum conditions for the reaction are a large excess of the diaminobenzene reagent in ethanol–acetic acid solution[100] or 2–4 *M* aqueous hydrochloric acid[101,102] at elevated temperatures. The quinoxalinol reaction is very selective and no interference from acetic, oxalic, lactic, citric, 2-hydroxybutyric, acetoacetic acids, acetone, glucose, fructose, ascorbic acid or dehydroascorbic acid at levels encountered in biological fluids was observed[103]. Under the conditions employed for quinoxalinol formation, the nitrite ion is converted into a benztriazole derivative[54] and selenium forms a piazselenole derivative[104]. Only two α-keto acids can not be determined as their quinoxalinol derivatives[102]. Oxaloacetic acid (and its methyl ester, oxaloacetate) undergo decarboxylation in acid solution to form pyruvic acid which does not form a stable quinoxalinol derivative. At low pH, *o*-hydroxyphenylpyruvic acid forms a stable δ-lactone which does not react with the diaminobenzene reagent. An enzymatic method has been developed for the decomposition of oxaloacetate prior to the determination of α-keto acids in tissue samples[101]. The aromatic α-keto acids (phenylpyruvic, *o*- and *p*-hydroxyphenylpyruvic and indolypyruvic acids) are unstable to atmospheric oxidation. This may be suppressed by the addition of sodium hydrogen sulfide[100] or 2-mercaptoethanol[105] to the reaction mixture.

Still some confusion remains concerning the structure of the derivative formed with 1,2-diaminobenzene and its subsequent analysis by GC after silylation. Hoffman and Killinger[106] have assigned the amide form to the trimethylsilyl derivative based on IR and NMR evidence. Frigerio *et al.*[107] could find no evidence for significant amide absorption in the IR spectra of the derivatives they prepared and likewise, using Fourier transform GC-IR, Langenbeck *et al.*[108] also failed to detect any amide absorption. This coupled with the prominent loss of *m/e* 89 [·OSi(CH$_3$)$_3$] in the mass spectrum of the quinoxalinol trimethylsilyl ether[107–109] tends to favor the phenolic tautomer structure. Langenbeck *et al.*[109] has rationalized the evidence in terms of the pH of the silylation reaction solution which they claim favors quinoxalinol TMS ether derivative formation.



To effect changes in chromatographic resolution and to increase sensitivity to selective GC detectors, several analogues of 1,2-diaminobenzene have been used. These are summarized below,



$R_3 = R_5 = R_6 = H, R_4 = NO_2$	1,2-diamino-4-nitrobenzene[103,110]
$R_3 = R_5 = R_6 = H, R_4 = Cl$	1,2-diamino-4-chlorobenzene[101,107]
$R_3 = R_5 = R_6 = H, R_4 = F$	1,2-diamino-4-fluorobenzene[101]
$R_3 = R_5 = R_6 = H, R_4 = CH_3$	2,3-diaminotoluene[100]
$R_3 = R_6 = H, R_4 = R_5 = Cl$	1,2-diamino-4,5-dichlorobenzene[107]
$R_3 = R_4 = R_5 = R_6 = F$	1,2-diaminotetrafluorobenzene[107]
	2,3-diaminonaphthalene[105]

The quinoxalinol derivatives of α-keto acids can be separated by paper chromatography[100,101,103,110–112] and by TLC and column chromatography[110]. For the development of paper chromatograms, alcoholic solvents containing various amounts of aqueous ammonia were preferred[100,103,112] as solvent mixtures of this type produced compact spots with the minimum of streaking. The use of fast-flow paper and spraying with sodium hydroxide prior to development gave improved chromatographic behaviour of the quinoxalinol derivatives[100]. Quinoxalinol derivatives were detected by their fluorescence in long-wavelength UV light with a detection limit in the region of 0.05–0.02 μmole[100,101]. For visual detection, the chromogenic reagent 1,2-diamino-4-nitrobenzene was introduced[103,110]. The quinoxalinol derivatives were weakly yellow in color and faded rapidly when removed from the TLC tank unless sprayed with potassium hydroxide solution. For quantitative analysis, elution of the spots with alkaline ethanol and spectrophotometric measurement of the eluted solution gave the best results[100,103]. Paper chromatography of the α-keto acid quinoxalinol derivatives has been used for their determination in *Penicillium chrysogenum*[113], rat urine[114] and human blood and urine[100,103].

Separation by GC of the quinoxalinol TMS ethers provides a rapid means of analysis giving complete separation of the α-keto acids of importance for studies of human metabolic dysfunction (with the exception of oxaloacetic and α-hydroxyphenylpyruvic acids)[102,109]. The methylene unit (MU) values of these quinoxalinol derivatives on the three stationary phases OV-1, OV-17 and Dexsil are summarized in Table 6. On Dexsil 300 GC, palmitic acid (generally found in urine) and carboxyethylquinoxalinol (from α-ketoglutaric acid) are well separated, whereas they are eluted as a single peak on OV-1. Branched chain α-keto acids are determined on OV-1 because *n*-propylquinoxalinol (from α-keto valeric acid) and isobutyl-1-quinoxalinol (from α-keto-β-methylvaleric acid) are well separated on this phase but not on Dexsil[102].

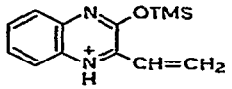
TABLE 6

METHYLENE UNIT VALUES OF TRIMETHYLSILYLQUINOXALINOLS

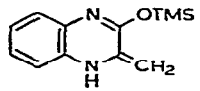
3% stationary phase loading on Gas-Chrom Q (100–120 mesh), nitrogen flow-rate 60 ml min^{-1} and temperature programme from 50 to 180° at 2° min^{-1}.

α-Keto acid	*MU Value*		
	OV-1	*OV-17*	*Dexsil 300 GC*
Pyruvic	15.13	16.57	15.41
Ketobutyric	15.78	17.17	16.06
Ketoisovaleric	16.00	17.21	16.20
Ketovaleric	16.46	17.84	16.73
Keto-β-methyl-*n*-valeric	16.65	17.90	16.81
Ketoisocaproic	16.77	18.07	16.95
Ketooctanoic	19.23	20.68	19.50
Keto-γ-(methylthio)butyric	19.48	21.83	20.11
Ketoglutaric	20.48	22.28	20.76
Phenylpyruvic	20.63	23.24	21.26
Ketoadipic	21.48	23.34	21.81

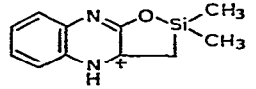
Using acetone deproteinization and cation-exchange chromatography as a sample pre-treatment method for biological fluids (urine, plasma, muscle tissue) as little as 0.005 m*M* of the α-keto acid quinoxalinol trimethylsilyl ether derivatives were determined with the flame ionization detector (FID)[115,116]. This compares with previous detection limits of 0.1–0.15 m*M* obtained for α-keto acids in urine without preliminary separation by cation-exchange chromatography[102]. To improve the sensitivity and selectivity with which the quinoxalinol derivatives can be determined, the AFID[114] and mass fragmentography[98,104,105] have been used. No detection limit was given for the AFID (rubidium sulphate disk) but it was stated that a calibration curve could be established for the range 4–40 mg l^{-1} of α-keto acids and this was adequate for the direct determination of α-keto acids in 50 ml of urine[117–119]. This corresponds to a sensitivity enhancement over the FID of about 50-fold[119]. Using mass fragmentography, 30–100 pmoles of the α-keto acids could be determined depending on the ion selected for analysis and the ion current distribution for the quinoxalinol trimethylsilyl ether derivative[102,108,109]. The technique is sufficiently sensitive for the determination of α-keto acids in 2.0-ml urine samples. The mass spectra of ten α-keto acids as their quinoxalinol TMS ether derivatives have been reported[108] and characteristic ions identified. One of the ions from the series *m/e* 217, 232 and 245 being present in nearly all derivatives studied were selected for mass fragmentography.



m/e 245



m/e 232

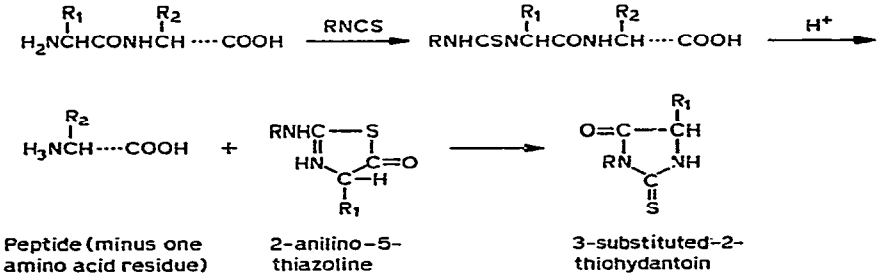


m/e 217

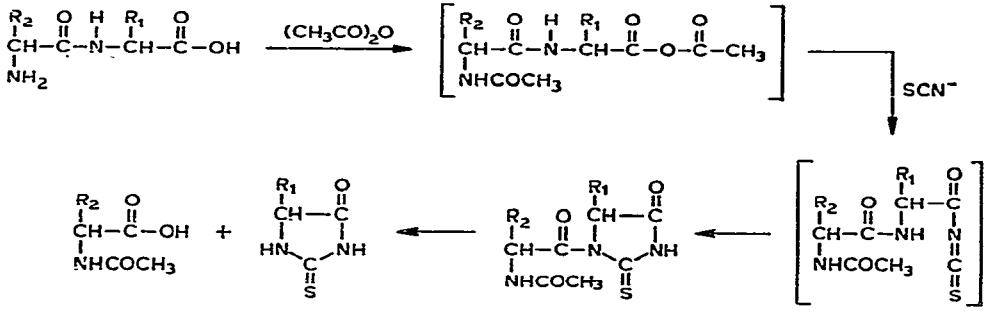
2,3-Diaminonaphthalene has been used for the selective analysis of phenylpyruvic acid in urine by reversed-phase HPLC[105]. Other α-keto acids such as pyruvic, 2-oxobutyric, 2-oxoglutaric and 4-hydroxphenylpyruvic acids did not interfere in the determination. Using UV absorption at $\lambda = 254$ nm the phenylpyruvic acid derivative could be easily detected below the μg level of derivative injected on-column.

7. THIOHYDANTOIN DERIVATIVES OF AMINO ACIDS

The Edman degradation procedure is commonly used for the sequential analysis of the amino acid composition of proteins and peptides. The free amino group of the peptide is reacted with an organic isothiocyanate to form a thiocarbamoyl peptide derivative which is subsequently cleaved under acid conditions to liberate the 2-anilino-5-thiazolinone derivative and the original peptide less the terminal amino acid group. The 2-anilino-5-thiazoline derivative is rearranged under acid conditions to the more stable cyclic thiohydantoin derivative which is separated from the peptide and used to identify the terminal amino acid group by one of several chromatographic techniques. The whole procedure can be automated and sequential peptide analyzers are commercially available. The chemical steps in the procedure are shown schematically below:



Although less frequently applied, sequencing of peptides from the carboxylic acid end group can be achieved in a similar manner to that described for the amine end group. The procedure, shown below, was first described by Schlack and Kumpf[120] and developed as a method of sequencing by Cromwell and Stark[121] and Yamashita[122]. The method is applicable to all polypeptides having a free carboxylic acid group and a free or monosubstituted α-amino group. The procedure described by Yamashita employs an ion-exchange resin to cleave the peptidyl thiohydantoin bond and does not promote cleavage of the parent peptide bonds observed sometimes with the acid conditions described by Cromwell and Stark.



A full discussion of the Edman procedure is beyond the scope of this review. Recent reviews by Edman[123], Niall[124], Rosmus and Deyl[125,126] and Deyl[127] cover this topic adequately. The reviews by Rosmus and Deyl[125–127] appeared in this journal

and to avoid unnecessary duplication only a brief summary of the chromatographic separation of thiohydantoins will be given here.

7.1. 2-Thiohydantoins

The 2-thiohydantoin derivatives of the amino acids can be identified by TLC on silica gel[121,122,128] and polyamide sheets[129]. Usually at least two solvent systems are necessary for the unequivocal identification of the protein amino acid derivatives on silica gel[121]. For the identification of thiohydantoin amino acid derivatives on polyamide plates, two-dimensional development of the TLC plate is employed with acetic acid–water (7:13) in the first direction and chloroform–95% ethanol–acetic acid (20:10:3) in the perpendicular direction. All common protein amino acid derivatives can be separated except for glutamine and glutamic acid (the latter may arise by hydrolysis of the former under the experimental conditions). The thiohydantoins strongly quench fluorescence in UV light and can be detected at the 50–100-nmole level on silica gel plates[121,122] and at the 0.5-nmole level on polyamide sheets[129]. The amino acid thiohydantoin derivatives produce a range of characteristic colors when sprayed with ninhydrin solution and this aids the confirmation of their identity[128].

The thiohydantoin derivatives of alanine, valine, leucine, isoleucine and methionine are sufficiently volatile to be separated by GC without derivatization[121]. Early attempts to prepare TMS and methyl derivatives of the remaining amino acid thiohydantoins resulted in the formation of doublet peaks on GC. Formation of the TMS derivatives in pyridine–BSTFA (1:1) at 50° for 10 min gave two peaks for glycine (mono- and bis-TMS derivatives), serine and threonine (side-chain dehydration) as well as causing the racemization of isoleucine to D-*allo*-isoleucine[130]. With a mixture of ethyl acetate–BSA (1:1) and heating at 80° for 5 min doublet peaks were obtained for glycine, threonine, asparagine, carboxymethylcysteine and pyridylethylcysteine thiohydantoins[128]. Fourteen TMS-thiohydantoin derivatives were separated and identified on a 4-ft. column of 10% SP-400 on Chromosorb W HP in less than 30 min. The derivatives of aspartic acid, methionine, glutamic acid, phenylalanine and asparagine were not resolved to baseline and the peaks for histidine, lysine and tyrosine were poorly separated. Mass spectra of the TMS-thiohydantoin amino acid derivatives have been recorded[130,131].

By using three isocratic solvent systems at 62°, sixteen thiohydantoin amino acid derivatives were identified by reversed-phase HPLC[132]. The derivatives of valine and tyrosine co-elute with a mobile phase of 0.01 *M* sodium acetate (pH 4.5) but are separated when the mobile phase contains 24% acetonitrile. Complete resolution of the hydrophobic (valine, isoleucine, leucine) and aromatic (phenylalanine, tryptophan) amino acid derivatives was obtained with a mobile phase consisting of 0.01 *M* sodium acetate (pH 4.5) and 10% acetonitrile.

7.2. 3-Methyl-2-thiohydantoins

The mobility (R_F values) of nineteen methylthiohydantoin (MTH) amino acid derivatives on polyamide sheets with two solvent systems has been described[133]. Neither solvent was able to separate leucine and isoleucine MTH derivatives. 0.05–0.1 nmoles of the MTH-amino acid derivatives could be detected by fluorescence quenching in UV light.

All the common protein MTH-amino acid derivatives except for aspartic acid,

arginine, serine, threonine, cysteic acid, S-carboxymethylcysteine and glutamic acid are sufficiently volatile to be separated by GC without derivatization[134]. Methylene unit values for the stable MTH-amino acid derivatives are given in Table 7[137]. A complete separation of all the MTH-amino acids of interest for protein sequencing has not been achieved by GC and the decomposition of some MTH derivatives produces products interfering with the identification of others (some MTH-amino acid derivatives decompose on metal columns)[136]. To improve separation characteristics and to reduce column adsorption of the MTH-amino acids the trimethylsilyl derivatives have been prepared[134,135,137–139]. Conditions employed for the formation of TMS-MTH-amino acid derivatives are BSTFA–acetonitrile (1:1.02) at 80° for 5 min[138], BSA–ethyl acetate (1:1) at 187° for 30 sec or 50° for 10 min[134], dissolution at room temperature in acetonitrile–BSA (3:1)[139] or acetonitrile–BSA (3:1) at 100° for 10 min[135]. Of the common protein amino acids the MTH derivative of glycine was converted to the mono-TMS derivative, tryptophan was incompletely silylated and the TMS-MTH derivatives of aspartic acid, glutamine, ornithine, lysine and histidine were partly degraded on the GC column[138]. Nineteen TMS-MTH-amino acid derivatives could be separated by temperature program analysis in less than 1 h[138]. Eighteen TMS-MTH-amino acid derivatives were separated by temperature program analysis on OV-17 as stationary phase. The derivatives of phenylalanine and asparagine co-elute in this system[135]. Retention index values for the TMS-MTH protein amino acids are given in Table 8. MS shows that silylation occurs on the thiohydantoin ring in all cases as well as on the side chain functional groups[139].

The FPD in the sulfur mode can be used to improve the selectivity and sensitivity of detection of the TMS-MTH-amino acid derivatives[140]. Detection limits in the range 0.07–0.039 nmoles were obtained. The TMS-MTH derivatives of threonine,

TABLE 7

METHYLENE UNIT VALUES FOR THE MTH DERIVATIVES OF THE COMMON PROTEIN AMINO ACIDS ON TWO STATIONARY PHASES[137]

6 ft. × 0.25 in. I.D. glass columns. Nitrogen flow-rate 90 ml min^{-1}.

MTH-Amino acid	*MU Value*	
	5% SE-30	*3% OV-17*
Glycine	14.63	19.08
Valine	15.12	18.68
Proline	15.49	19.95
Threonine	15.75	19.64
Alanine	15.81	20.82
Isoleucine	16.14	19.78
Leucine	16.26	19.81
Glutamic acid	17.80	23.70
Methionine	18.88	23.76
Phenylalanine	19.61	24.88
Asparagine	21.28	26.35
Glutamine	22.00	28.00
Lysine	23.07	29.98
Tyrosine	23.17	29.47
Histidine	23.78	30.16
Tryptophan	26.77	34.25

TABLE 8

RETENTION INDICES FOR THE TMS-MTH COMMON PROTEIN AMINO ACIDS ON TWO STATIONARY PHASES

TMS-MTH-Amino acids	*Column temperature (°C)*	*Retention index*	
		*SE-30**	*OV-17***
Alanine	140	1491	1724
Valine	140	1561	1757
S-Carboxymethylcysteine	140	1546	1773
Isoleucine	140	1649	1837
Leucine	140	1658	1857
Δ-Threonine	140	1582	1905
Proline	140	1543	1905
Glycine	140	1611	1764
Aspartic acid	180	1896	2140
Methionine	180	1912	2203
Glutamic acid	180	2016	2246
Phenylalanine	180	2005	2307
Asparagine	180	2016	2307
Glutamine	180	2154	2447
Tyrosine	220	2343	2616
Histidine	220	2298	2645
ε-MTC-Lysine	220	2326	2721
Tryptophan	220	2631	2926

* 175 × 0.4 cm I.D. glass column packed with 1.70% SE-30 on Supelcoport (80–100 mesh).
** 175 × 0.4 cm I.D. glass column packed with 1.70% OV-17 on Supelcoport (80–100 mesh).

glycine, asparagine, glutamine, proline, histidine and *ε*-MTC-lysine gave low responses to the FPD when less than 2.5 nmoles were chromatographed.

Isolation of the thiazoline intermediate of asparagine and glutamine was used to distinguish these two amino acids from aspartic acid and glutamic acid which are formed under the acid conditions required for cyclization to the thiohydantoin derivative. The amino acids are identified by GC–MS after formation of their TMS derivatives[141].

7.3. 3-Phenyl-2-thiohydantoins

Silica gel TLC has often been used for the identification of phenylthiohydantoin (PTH) amino acid derivatives. For the selection of useful solvent systems, the reviews mentioned at the beginning of this section should be consulted. Usually two solvent systems are necessary for the unequivocal identification of a PTH-amino acid. One solvent system is used to separate the polar PTH-amino acid derivatives and a second solvent system to separate derivatives with similar mobilities (*e.g.* leucine/isoleucine, valine/phenylalanine)[142]. Spraying the plate after development with ninhydrin produces characteristic colors for the different PTH-amino acid derivatives which can be used to aid identification[143]. The use of micro silica gel TLC plates (6.3 × 6.3 cm) has been recommended as providing an increase of 10–20-fold in the speed of analysis[144]. It was also claimed that aluminium backed silica gel plates are superior to glass plates giving more compact spots and better resolution[145,146]. Nearly all the PTH derivatives of the common protein amino acids can be identified in a

short time by two-dimensional development on polyamide layers. Twenty-one PTH-amino acid derivatives were identified with the solvent systems ethylene chloride–glacial acetic acid (90:16) and toluene–pentane–glacial acetic acid (60:30:20)[147]. Leucine/isoleucine and methionine/methionine sulfone were not separated. Sixteen out of twenty PTH-amino acid derivatives were resolved using toluene–pentane–glacial acetic acid (60:30:35) and 35% aqueous acetic acid as solvent systems[148]. The relative mobility of the PTH derivatives was found to be very reproducible in this system and representative R_F values are summarized in Table 9. Twenty-four PTH-amino acid derivatives could be identified in less than 30 min with a two-dimensional development using toluene–pentane–acetic acid (60:30:16) and either 25% aqueous acetic acid or 40% aqueous pyridine–acetic acid (9:1) as the second solvent[149]. The addition of a fluorescence indicator to the polyamide layer or developing solvent enabled 0.05–0.2 nmoles of the PTH-amino acid derivatives to be detected in UV light[148,149].

TABLE 9

R_F VALUES FOR THE COMMON PROTEIN AMINO ACIDS IN TWO SOLVENT SYSTEMS ON 5 × 5 cm POLYAMIDE SHEETS

PTH-Amino acid	*R_F Value*	
	Toluene–pentane–acetic acid (60:30:35)	*35% Aqueous acetic acid*
Alanine	0.63 ± 0.01	0.52 ± 0.02
Arginine	0.63 ± 0.01	0.95 ± 0.02
Asparagine	0.31 ± 0.02	0.63 ± 0.01
Aspartic acid	0.28 ± 0.02	0.46 ± 0.01
Cysteic acid	0.02 ± 0.00	0.13 ± 0.01
Glutamine	0.54 ± 0.01	0.60 ± 0.02
Glutamic acid	0.37 ± 0.02	0.49 ± 0.01
Glycine	0.56 ± 0.02	0.57 ± 0.01
Histidine	0.03 ± 0.01	0.95 ± 0.02
Isoleucine	0.82 ± 0.02	0.33 ± 0.01
Leucine	0.82 ± 0.02	0.33 ± 0.01
Lysine	0.45 ± 0.02	0.16 ± 0.00
Methionine	0.71 ± 0.03	0.38 ± 0.01
Phenylalanine	0.74 ± 0.03	0.28 ± 0.01
Proline	0.87 ± 0.03	0.51 ± 0.01
Serine	0.26 ± 0.02	0.61 ± 0.01
Threonine	0.39 ± 0.02	0.59 ± 0.01
Tryptophan	0.38 ± 0.02	0.16 ± 0.00
Tyrosine	0.18 ± 0.01	0.26 ± 0.01
Valine	0.79 ± 0.02	0.40 ± 0.01

The PTH-amino acid derivatives of alanine, glycine, valine, leucine, isoleucine, methionine, proline and phenylalanine are sufficiently volatile to be gas chromatographed without further derivatization[150]. Other PTH-amino acid derivatives were either unstable or exhibit poor chromatographic properties. The formation of TMS derivatives improves the general chromatographic properties of the PTH-amino acids markedly[150–152]. PTH-proline does not form a TMS derivative and PTH-lysine and PTH-arginine do not form TMS derivatives stable to GC. PTH-cystine and PTH-

cysteine form TMS derivatives which do not correspond to simple derivatives. The PTH derivatives of serine, threonine, tyrosine, glutamine, asparagine, histidine and tryptophan are stable to GC after formation of their TMS derivatives. The relative retention times of some PTH-amino acids and their TMS derivatives are summarized in Table 10[157]. Combined GC–MS indicates that silylation occurs on the nitrogen atom of the hydantoin ring as well as the expected side-chain functional groups, the imidazole ring of histidine and the phenol group of tyrosine[151,153]. The separation of all the common protein amino acid TMS-PTH derivatives on packed columns is difficult to achieve and usually some overlaps exist between different pairs of amino acid derivatives depending on the stationary phase selected for the separation. With the FPD (sulfur mode), nanogram amounts of the TMS-PTH amino acid derivatives were detected[152].

TABLE 10

RELATIVE RETENTION TIMES OF PTH-AMINO ACIDS AND THEIR TMS DERIVATIVES ON SE-30[157]

Amino acid	*PTH derivative (170°C)*	*TMS-PTH derivative*
Alanine	0.79	1.03
Glycine	0.94	1.05
Isoleucine	1.55	2.12
Leucine	1.65	1.98
Proline	1.38	1.38
Serine	0.89*	2.24
Threonine	0.97*	2.76
Valine	1.12	1.41
	(200°C)**	
Asparagine		5.53
Aspartic acid		3.41
Glutamine		8.25
Glutamic acid		4.91
Methionine	3.72	3.86
Phenylalanine	5.00	5.23
	(225°C)***	
Tyrosine	1.13	1.03
Histidine	1.27	0.98
Tryptophan	2.59	2.13

* Dehydration product.
** Relative to androstane.
*** Relative to cholestane.

Acetyl and trifluoroacetyl derivatives of PTH-amino acids have also been prepared to improve their separation properties[154–156]. The acetyl derivatives are considerably less volatile than the trifluoroacetyl derivatives but their chromatographic properties were considered to be superior[155]. Unfortunately, a single set of experimental conditions could not be established for the formation of the acyl derivatives of all the PTH-amino acids important in protein sequencing[154,155].

The PTH-amino acid derivatives can be identified and quantified without chromatographic separation using chemical ionization MS with *tert.*-butane as reagent gas[157]. The PTH-amino acids are relatively strong bases and all have a stable

$[M+1]^+$ ion (except for lysine, SCM-cysteine and arginine) in their chemical ionization mass spectra which serves for their identification. Quantitation is achieved by using the d_5-labelled PTH-amino acid as internal standard.

Recent years have witnessed an explosive growth in the application of HPLC to the identification and quantitation of the PTH-amino acids originating from the protein sequenator[158–179]. The advantages of HPLC compared to other techniques are its high speed, ease of automation, high sensitivity and the fact that further derivatization is not required prior to analysis. As little as 5 pmoles of PTH-amino acid derivatives can be detected with the UV detector[164]. The PTH-amino acid derivatives differ widely in polarity and this has made the development of a single isocratic HPLC separation difficult. Chromatographic systems currently recommended for the separation of PTH-amino acids are summarized in Table 11. Many of the systems meet the requirement of being able to keep pace with the sequenator. Reversed-phase C_{18} columns with gradient elution using organically modified sodium acetate buffers are the most widely used separation method in HPLC for these derivatives.

7.4. Miscellaneous thiohydantoins

Pentafluorophenylisothiocyanate has been used instead of phenylisothiocyanate to form pentafluorophenylthiohydantoin (PFPTH) derivatives in the Edman procedure[180]. The PFPTH-amino acids have good physical properties, can be identified by TLC and are significantly more volatile on GC than the PTH-amino acids.

p-Bromophenylisothiocyanate has been used in conjunction with MS to better identify PTH-amino acid derivatives without the aid of chromatographic separation[181]. The characteristic doublet for the bromine isotope peaks in the electron-impact mass spectra of the *p*-BrPTH derivatives simplifies identification based on the presence of a molecular ion.

p-Phenylazophenylisothiocyanate forms colored derivatives with amino acids obtained by the Edman procedure. All the *p*-phenylazophenylthiohydantoins (PAPTH) of the common protein amino acids can be identified within 30 min using silica gel TLC and two solvent systems[182]. Spraying the PAPTH amino acids with different reagents resulted in the development of characteristic colors which could be used to confirm the identity of a derivative when used in conjunction with R_F data[183].

The terminal carboxylic acid group of peptides can be sequenced by formation of its iminohydantoin derivative, a few of which have been separated by TLC and GC[184].

4-N,N-Dimethylaminoazobenzene-4'-isothiocyanate reacts with amino acids liberated during sequence analysis and the derivatives identified by two-dimensional TLC on polyamide sheets[185]. The colored derivatives were detected down to the picomole level directly on the polyamide sheet.

8. CYCLIC SILICON-CONTAINING DERIVATIVES

Dimethyldichlorosilane (DMCS) in pyridine was used to form a cyclic siliconide derivative of 3β-acetoxy-16α,17α-dihydroxypregn-5-en-20-one and its mass spectrum recorded[186]. DMCS in pyridine was also used to form a siliconide derivative of 3-methyl-18-hydroxylestradiol-17β (the 17α analogue does not form a derivative)[187]. The principle problem in using DMCS as a reagent is that polyfunctional steroids con-

TABLE 11

CONDITIONS USED IN HPLC FOR THE SEPARATION OF PTH-AMINO ACIDS

FR = Flow-rate.

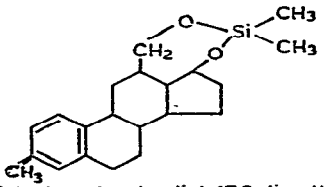
Stationary phase	*Method of operation*	*Mobile phase*	*Comments on separation*	*Ref.*
Adsorption				
1.0 m × 1.8 mm I.D. 15–20 μm silica	Isocratic FR = 1.0 ml min^{-1} 25°	(a) C_7H_{16}–$CHCl_3$ (1:1) (b) $CHCl_3$ (c) CH_3OH–$CHCl_3$ (3:97)	(a) Met, Phe, Val, Ile, Leu, Pro separated in 16 min (b) Ala, Gly, Tyr, Thr separated in 28 min (c) Asn, Gln, Ser separated in 12 min	158
50 cm × 0.3 mm I.D. Merckosorb SI 60	Isocratic FR = 1.65 ml min^{-1}	(a) CH_2Cl_2–DMSO–*tert.*-C_4H_9OH (100:0.8:8) (b) CH_2Cl_2–DMSO–H_2O (180:15:2) (c) CH_2Cl_2–*tert.*-C_4H_9OH (1000:8)	(a) Pro, Leu, Ile, Val, Phe, Met, Ala, Trp, Gly separated in 7 min (b) Tyr, Lys, Thr, Glu, Gln, Asn, Asp separated in 7 min, Gly/Gln, Tyr/Thr poorly separated (c) Leu, Ile and 7 other PTH-amino acids separated in 9.5 min	159
50 cm × 0.2 cm I.D. Merckosorb SI 60	Isocratic FR = 0.5 ml min^{-1}	CH_2Cl_2–iso-C_3H_7OH (1000:8)	Pro, Leu, Ile, Val, Phe, Met, Trp, Ala, Gly separated in 17 min	160
25 cm × 2.1 mm I.D. Zorbax Sil, 8 μm	Concave gradient FR = 0.6 ml min^{-1}	C_6H_{14}–CH_3OH–C_3H_7OH (3980:9:11) to CH_3OH–C_3H_7OH (9:11)	All PTH-amino acids (except His, Arg) identified in 40 min. Gly/Thr, Lys/Tyr co-elute. Lys, Thr identified from by-products	161
30 cm × 3.9 mm I.D. Porasil, 10 μm	Concave gradient FR = 1.5 ml min^{-1}	(a) A: iso-C_8H_{18}; B: CH_3OH–iso-C_3H_7OH (9:11); 5 → 70% B (b) A: iso-C_8H_{18}–$CHCl_3$; B: CH_3OH–C_8H_{18}–$CHCl_3$ (15:42.5:42.5); 1 → 1004% B	(a) Leu/Ile, Phe/Met, Glu/Asp co-elute. Val/Pro, Gly/Thr/Trp not separated to baseline. 20 min (b) Trp/Lys co-elute. Trp/Gly, Tyr/Thr, Met/Phe not separated to baseline. 20 min	162
Partition				
2.5 cm × 2.1 mm I.D. Micropak-CN, 10 μm	Step gradient FR = 0.83 ml min^{-1}	A: C_6H_{14} B: CH_2Cl_2–iso-C_3H_7OH (1:1)	17 PTH-amino acids separated. Poor column efficiency. Many overlaps. 40 min	163

(Continued on p. 128)

TABLE 11 *(continued)*

Stationary phase	*Method of operation*	*Mobile phase*	*Comments on separation*	*Ref.*
1 m × 2 mm I.D. Permaphase ETH, 30 μm	(a) Isocratic	(a) 0.01 *M* NaOAc	(a) His/Arg separated in 12 min. 5 other non-polar derivatives separated in 20 min	164
	(b) Exponential concave gradient	(b) A: 0.01 *M* NaOAc; B: CH_3CN; 10% B min^{-1}	(b) Ser/Thr/His/Arg co-elute. Tyr/Met, Trp/Lys, Gln/Asn poorly separated. 40 min	
50 cm × 2.1 mm I.D. Permaphase ETH	Linear gradient	A: H_2O; B: CH_3CN; A → 30% B at 2% min^{-1}	12 PTH-amino acids separated. 17 polar PTH derivatives separated	
Reversed-phase			C_{18}	
3 ft. × 2.0 mm I.D. Corasil C_{18}	Linear gradient FR = 0.8–1.2 ml min^{-1}	A: 0.01 *M* NaOAc (pH 3–8)–CH_3CN (95:5); B: CH_3CN; 2% B min^{-1}	Few PTH-amino acids separated to baseline. 30 min	165
1 m × 4.0 mm I.D. Corasil C_{18}	Step gradient FR = 1.0–1.2 ml min^{-1}	H_2O–CH_3CN–iso-C_3H_7OH (100:1.5:1) to (15:1.5:1)	Poor column efficiency. Many overlaps	166
60 cm × 4.0 mm I.D. Bondapak C_{18}	3 gradient programs	A: 0.01 *M* NaOAc (pH 4.0)–CH_3CN (9:1); B: 0.01 *M* NaOAc (pH 4.0)–CH_3CN (1:9)	20 PTH-amino acids identified by a choice of 3 systems using two buffers	167
25 cm × 2.0 mm I.D. Zorbax ODS, 5–6 μm	Linear gradient FR = 0.5 ml min^{-1} 50°	A: 0.01 *M* NaOAc (pH 5.0)–CH_3CN (95:5); B: CH_3CN; 1% B min^{-1}	All common PTH-amino acids separated except Gly/Gln, Met/Val which co-elute. Phe/Leu/Ile poorly separated	164
25 cm × 4.6 mm I.D. LiChrosorb C_{18}	3 gradient programs	A: 0.1 *M* NaOAc (pH 4.6) at 82°; B: CH_3CN at 47°	All common PTH-amino acids separated in 3 programmed gradient runs	168
25 cm × 4.6 mm I.D. Partisil ODS, 10 μm	Isocratic FR = 2–3 ml min^{-1}	0.01 *M* NaOAc (pH 4.0) with 10, 20 and 30% CH_3CN	21 PTH-amino acids separated in 3 isocratic runs	169
25 cm × 4.6 mm I.D. Zorbax ODS	Gradient FR = 1.0 ml min^{-1}	A: 0.01 *M* NaOAc (pH 4.5); B: CH_3CN	Under isocratic conditions A–B (42:58) all PTH-amino acids except Ser/Gln which co-elute are separated in 15 min. 20-PTH-amino acids separated by gradient elution. 20 min. Ser, Gln separated in a separate system.	170

30 cm × 3.9 mm I.D. μBondapak C_{18}, 10 μm	Step gradient	A: 0.005 *M* NaOAc (pH 5.3)–acetone (77:23) + 20 μl CH_3OH; B: as above, 44% CH_3OH C: as A, 22% CH_3OH–5% CH_3COOH	17 PTH-amino acids separated in A → 100% B. Val/Met, Phe/Ile co-elute. 16 min Solvent C separates Cys, His, Arg in 30 min isocratically	171
30 cm × 44 mm I.D. Bondapak C_{18}	Linear gradient FR = 2.5 ml min^{-1}	A: H_2O–CH_3OH–CH_3CO_2H (900:100:2.5) + 50 μl $(CH_3)_2CO$; B: H_2O–CH_3OH–CH_3CO_2H (100:900:0.25); 95% A + 5% B to 55% A + 45% B	21 PTH-amino acids tested. Val/Met co-elute. Leu/Ile/Phe poorly separated. 20 min	172
2 × [15 cm × 3.9 mm I.D.] LiChrosorb RP-8, 5 μm	Isocratic FR = 0.9 ml min^{-1} 50°	0.01 *M* NaAOc (pH 4.6) (4:1)	21 PTH-amino acids separated. Met/Pro co-elute	173
30 cm × 4.0 mm I.D. Bondapak C_{18}	Concave gradient + Isocratic period	A: 0.01 *M* NaOAc (pH 4.0)–CH_3OH (9:1); B: 0.01 *M* NaOAc (pH 4.0)–CH_3OH (1:9); 5% B → 40% B	All common PTH-amino acids separated except Met/Val. 32 min	174
30 cm × 4.0 mm I.D. μBondapak C_{18}	4 gradient programs used	A: 0.01 *M* NaOAc (pH 4.0)–CH_3CN (9:1); B: as above (1:9)	(a) Ala, Tyr, Val, Ile, Lys separated in 20 min. (b) Asp, Asn, Ser, Thr, Gly, Gln, S-CMC separated in 15 min (c) Pro/Met, Trp/Phe separated isocratically (d) Cys, His, Arg separated in 8.0 min. 22 PTH-amino acids can be identified with 2 injections	175
30 cm × 4.0 mm I.D. Bondapak C_{18}	Concave gradient FR = 4.0 ml min^{-1}	A: 8.0 m*M* Diethylenetetramine + 20.0 m*M* trichloroacetic acid pH 4.2; B: as above + 60% CH_3CN	Trp/Phe/Leu and Pro/Val co-elute. Only a few separations to baseline. 40 min	176
Bonded peptide stationary phases				
25 cm × 3.8 mm I.D. L-Val-L-Ala-L-Ser bonded onto silica CT	Isocratic	1% Citric acid–water pH 2.5	15 PTH-amino acids separated well enough for identification purposes	177
25 cm × 2.1 mm I.D. L-Val-L-Phe-L-Val bonded onto Partisil 10 0.26 mmole g^{-1} peptide	Isocratic	1% Aqueous citric acid (pH 2.5)–CH_3OH (20:1)	11 PTH-amino acids separated with good selectivity but column efficiency is poor	178

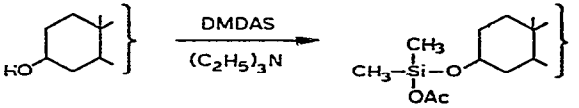


3-methyl-18-hydroxyloestradiol-17β dimethylsiliconide

taining isolated functional groups (ketones as well as hydroxyl groups) react to various extents yielding thermally unstable products. Corticosteroids with an 11β-OH group gave unstable products due to partial reaction of the hydroxyl group with DMCS[188]. The siliconide derivative of the corticosteroid side chain was very moisture sensitive and was formed in about 40% yield when DMCS pyridine was used as the reaction medium. For corticosteroids with isolated functional groups (*e.g.*, cortisol) a mixed derivative has been prepared using dimethylmethoxychlorosilane (DMMCS) to form the side chain siliconide derivative and to simultaneously catalyze the reaction of 1,1,3,3-tetramethyldisilazane (TMDS) with the hindered 11β-OH group[188]. The yield

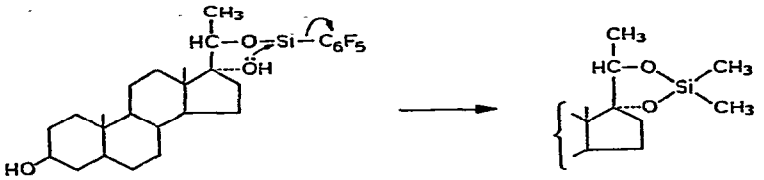
Cortisol

of mixed derivative was about 10% and the reaction is limited to steroids with hindered hydroxyl groups. Steroids with unhindered hydroxyl groups such as 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione react with the mixed reagent to form multiple products unsuitable for GC. To avoid this problem, dimethyldiacetoxysilane (DMDAS) was introduced[189]. This reagent forms identical siliconide derivatives with diols and with isolated hydroxyl groups forms thermally stable dimethylacetoxy derivatives. The DMDAS reagent has moderate silyl donor power, reacting smoothly with unhindered hydroxyl groups in the presence of base catalysis but not significantly with 11β and more sterically hindered hydroxyl groups. Reactions are carried out in dilute solution to minimize the formation of derivatives with silyl bridges (the diacetoxy group reacting with two steroid molecules). The cyclic dimethylsiliconide derivatives were formed in 75–95% yield with *cis*-diols and steroids with the dihydroxyacetone side chain. In a separate study, dimethylsiliconides were formed satisfactorily with steroids containing a dihydroxyacetone side chain and 17α,20-diols but not from 20,21-diols[190]. Both the reagent DMDAS and the derivatives were extremely susceptible to hydrolysis.

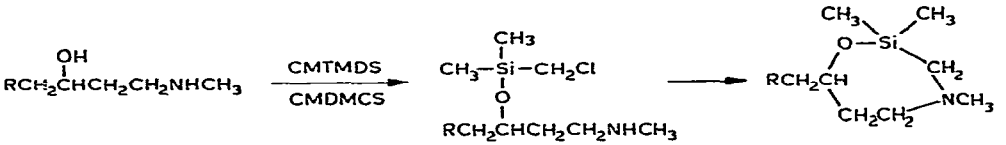


Dimethylacetoxy derivative

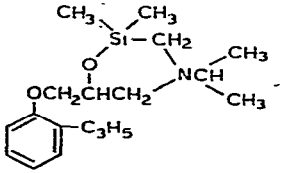
A dimethylsiliconide derivative was formed as an unexpected product from the reaction of flophemesylamine with 5β-pregnane-17α,20-diol[191]. Presumably the bulky flophemesyl reagent reacts preferentially with the exposed 20-hydroxyl group and the dimethylsiliconide derivative is formed by nucleophilic attack of the 17α-OH group at the silicon center with expulsion of pentafluorobenzene.



β- and γ-hydroxylated primary, secondary, tertiary and quaternary amines and possibly some enolizable ketone groups react with a mixture of 1,3-bis(chloromethyl)-1,1,3,3-tetramethyldisilazane (CMTMDS) and chloromethyldimethylchlorosilane (CMDMCS) to form cyclic derivatives[192]. The reaction takes place smoothly at room temperature or upon heating to 60° for 5–15 min. Under these conditions the amine



group is not derivatized and reaction initially takes place at the hydroxyl group followed by cyclization in solution involving elimination of chlorine as hydrogen chloride from the chloromethyl group. The reaction with phenacylamine (β-keto amine) was incomplete and although phenylalanine gave a cyclic derivative, other α-amino acids (tryptophan, proline) gave poor results. β-Hydroxy quaternary amino compounds may not react to completion and produce by-products on GC. The cyclic silicon derivatives have good GC properties and their mass spectra were characterized by prominent molecular ions and diagnostically informative fragmentation pathways. The metabolism of the β-adrenoceptor antagonist drug, alprenolol in man and dog was followed by formation of a cyclic silicon derivative of the side chain using the method described above[193]. The low level of drug and its metabolites in biological fluids were determined by mass fragmentography.



Cyclic Dimethylsilyl Derivative of Alprenolol

The dimethylsilyl derivative was found to be stable to enzymatic hydrolysis and to the conditions necessary for forming TMS derivatives of hydroxylated metabolites with trimethylsilylimidazole. One metabolite of alprenolol, resulting from hydroxylation of the allylic substituent to a 1,2-diol, gave the normal bis(chloromethyldimethylsilyl)

ether and not the cyclic dimethylsilyl derivative under the usual reaction conditions for the formation of the cyclic derivative. Compounds which have been separated by GC as their cyclic silicon derivatives are summarized in Table 12.

TABLE 12

COMPOUNDS FORMING CYCLIC SILICON-CONTAINING DERIVATIVES USED IN GC

Compound	*Reagents*	*Comments*	*Ref.*
3β-Acetoxy-16α,17α-dihydroxy-pregn-5-en-20-one	DMCS	MS	186
Cortisone	DMCS	GC	188
	DMDAS	GC	189
Cortisol	DMMCS–TMDS	GC, MS	188
	DMDAS	GC	189
Tetrahydrocortisol	DMDAS	GC	189
Betamethasone	DMMCS–TMDS	GC, MS	188
	DMDAS	GC	189
3-Methyl-18-hydroxyoestradiol	DMCS	GC	187
17α,21-Dihydroxypregn-4-ene-3,20-dione	DMDAS	I_{OV-1}, MS	190
5β-Pregnane-3α,17α,20β-triol	DMDAS	$I_{dex.}$ I_{OV-1}, MS	190
	Flophemesylamine	$I_{dex.}$ GC, MS	191
Ephedrine	CMDMCS–CMTMDS	GC, MS	192
1-Phenyl-2-methylaminoethanol	CMDMCS–CMTMDS	GC, MS	192
Terbutaline*	CMDMCS–CMTMDS	GC	192
Propanolol**	CMDMCS–CMTMDS	GC, MS	192
Isoprenaline***	CMDMCS–CMTMDS	GC, MS	192
Aprobit§	CMDMCS–CMTMDS	GC, MS	192
Phenacylamine	CMDMCS–CMTMDS	GC	192
Phenylalanine	CMDMCS–CMTMDS	GC, MS	192
Clobutinol§§	CMDMCS–CMTMDS	GC, MS	192
Alprenolol§§§	CMDMCS–CMTMDS	GC, MS	193
4-Hydroxy-alprenolol	CMDMCS–CMTMDS	GC, MS	193
1′-Hydroxy-alprenolol	CMDMCS–CMTMDS	GC, MS	193
2′,3′-Dihydroxy-alprenolol	CMDMCS–CMTMDS	GC, MS	193

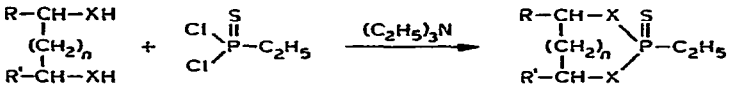
* 2-*tert.*-Butylamino-1-(3,5-dihydroxyphenyl)ethanol.
** 1-Isopropylamino-3-(1-naphthyloxy)-2-propanol.
*** 1-(3,4-Dihydroxyphenyl)-2-isopropylaminoethanol.
§ 10-[2-2(2-Hydroxyethyldimethylamino) propyl]-phenothiazine chloride.
§§ 4-Dimethylamino-1-(4-chlorophenyl)-2,3-dimethyl-2-butanol.
§§§ 1-Isopropylamino-3-(2-allylphenoxy)-2-propanol.

The reaction of organotrialkoxysilanes with trialkanolamines results in the formation of C-substituted silatranes (2,8,9-trioxa-5-aza-1-silatricyclo[3,3,3^{1,5}]-undecanes)[194]. These are polar intramolecular complexes with a transannular N→Si bond. Although of high boiling point, they are sufficiently stable to be separated by GC.

9. CYCLIC ETHYLPHOSPHONOTHIOIC DERIVATIVES

Ethylphosphonothioic dichloride (EPTD) reacts with bifunctional compounds

containing OH, NH_2 and CO_2H groups in the presence of triethylamine to form cyclic ethylphosphonothioic derivatives according to the following equation[195]:



Stable derivatives were also formed by *ortho* substituted (but not *meta* and *para*) bifunctional aromatic compounds and enolizable diketones. Dicarboxylic acids, α-hydroxyacids or aliphatic bifunctional compounds in which $n \geqslant 2$ did not form derivatives stable to GC. Although the rate of reaction depends on the steric arrangement and the chemical nature of the bifunctional group (*ortho* substituted aromatic compounds react slower than their aliphatic equivalent), all reactions studied were complete when 1.0 ml of a 10 mmolar solution of the bifunctional compound in acetonitrile, 15 μl of ethylphosphonothioic dichloride and 33 μl of triethylamine were heated at 80° for 30 to 45 min in a nitrogen atmosphere. Retention index data for some representative cyclic ethylphosphonothioic derivatives are given in Table 13. The second derivative peaks formed with phenyl-1,2-ethanediol and *cis*-1,2-cyclohexanediol are most probably due to the formation of geometric isomers based on mass spectral evidence. The EPTD derivatives can be determined with high selectivity at trace levels using selective GC detectors. Detection limits obtained with several detectors for a derivative of pinacol are summarized in Table 14. The NPD can be used to determine picogram quantities of the EPTD derivatives. The mass spectra of the EPTD derivatives are characterized by a prominent molecular ion which fragments to produce stable phosphorus-containing daughter ions constituting a considerable amount of the total ion current of the mass spectrum. The base peak in many spectra was formed by the loss of the elements of ethylsulfide from the molecular ion. Analysis of the mass spectra of the diol derivatives indicated the presence of characteristic ions useful for their identification.

TABLE 13

RETENTION INDEX VALUES FOR THE CYCLIC ETHYLPHOSPHONOTHIOIC DERIVATIVES OF BIFUNCTIONAL COMPOUNDS

90 × 0.2 cm I.D. nickel column packed with 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen flow-rate = 30 ml min^{-1}.

Cyclic ethylphosphonothioic derivative	*Retention index (I_{OV-17})*		*Column temperature (°C)*
	1st Peak	*2nd Peak*	
Ethylene glycol	1563	—	110
1,3-Propanediol	1568	—	110
Pinacol	1639	—	110
Catechol	1733	—	130
3-Amino-1-propanol	1788	—	130
1,3-Propanediamine	1968	—	150
cis-1,2-Cyclohexanediol	1961	2020	150
o-Aminophenol	2042	—	150
Phenyl-1,2-ethanediol	2185	2221	170
o-Phenylenediamine	2385	—	170

TABLE 14

THE MINIMUM DETECTABLE QUANTITY (MDQ) OF THE EPTD DERIVATIVE OF PINACOL

Detector	*Pinacol (ng)*	*P (g sec^{-1}) ($\times 10^{-15}$)*	*S (g sec^{-1}) ($\times 10^{-10}$)*
Nitrogen–phosphorus detector	0.002	2.0	—
Flame photometric detector (P mode)	0.5	2000	—
Flame photometric detector (S mode)	1.0	—	2.0
Electron-capture detector	10.0	—	—

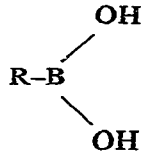
Ethyldichlorothiophosphate did not yield derivatives stable to GC under the reaction conditions used to prepare EPTD derivatives[195].

10. CYCLIC BORONIC ESTERS

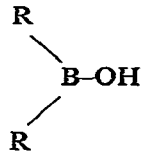
Since their introduction about twelve years ago by Brooks and Watson[209], the organic boronic acids have become well established as the most useful reagents for the chromatographic analysis of bifunctional compounds. They owe their popularity to their ability to react with a wide range of polar functional groups under mild conditions to give stable derivatives with good GC and MS properties. Recently, Poole *et al.*[282] have introduced several boronic acids with electron-capturing properties to extend the range of application of the boronic acids to the realm of trace analysis. The combination of the selective reaction of the boronic acid group, the separating power of the gas chromatograph and the high sensitivity and selectivity of the electron-capture detector promises a new vista for trace analysis of bifunctional compounds in complex mixtures with the minimum of sample manipulation.

10.1. General chemistry of boronic acids

The synthesis and properties of the boronic acids have been reviewed from the point of view of their basic chemistry and reactions[196–198]. A complete list of all boronic acids prepared up to about 1974 is available[198]. Boron forms two types of organoboron acids, the boronic acids and the borinic acids of general structure shown below:



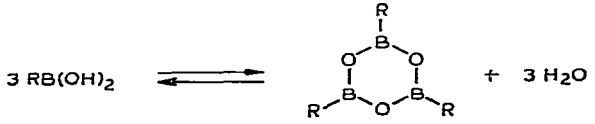
Boronic acid



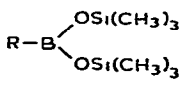
Borinic acid

The borinic acids have not been shown to have any analytical uses and will not be considered further.

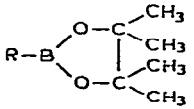
The boronic acids are very readily dehydrated to the trimeric cyclic anhydrides (boroxines) under mild conditions (*e.g.*, recrystallization). Most commercial samples



of boronic acids contain a variable amount of the anhydride. This is no disadvantage from the chromatographic point of view as the anhydride reacts as easily as the acid. The anhydride has good GC properties and is formed in the injection port when a solution containing a boronic acid is injected into the gas chromatograph. The anhydride is also formed when a boronic acid is heated in a melting point apparatus. Generally speaking, the determination of the melting point is not a good method of determining the purity of a boronic acid as the value obtained is dependent on the method and rate of heating. Complete dehydration of the acid by heating *in vacuo* over phosphorus pentoxide enables an accurate value for the anhydride to be obtained which can be used to characterize the original acid. The preparation of the highly crystalline and sharp melting diethanolamine derivatives are a more convenient method of characterization[199,200]. In a chromatographic laboratory, it is easier to characterize a boronic acid or to identify impurities in a sample of boronic acid by chromatographic techniques. On-column conversion of the boronic acid to the anhydride is too variable for quantitative analysis and the separation of boronic acids by TLC is difficult as the mobility of the acid is dominated by the boronic acid function and little influenced by the organic substituent. Boronic acids form bis-TMS esters which have good GC properties and characteristic mass spectra[201]. The molecular ions are generally weak but a prominent $[M-CH_3]^+$ ion can be used to identify the molecular weight. The bis-TMS ester derivatives are very hydrolytically unstable which makes their manipulation difficult. Pinacol can be used as a reagent to characterize boronic acids[202]. This has the advantage of producing crystalline derivatives in quantitative yield (with aromatic boronic acids) which are hydrolytically stable and can be submitted to TLC or HPLC.



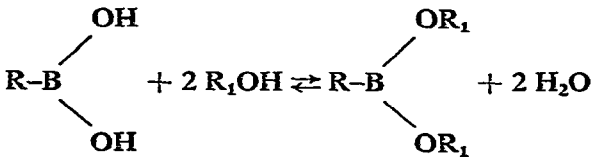
Boronic acid bis-TMS ester



Pinacol boronate

The pinacol boronates are volatile with good peak shape on GC and their mass spectra are distinguished by a strong molecular ion and characteristic modes of fragmentation reflecting the nature of the organic function of the boronic acid.

Boronic acids react with monofunctional compounds such as alcohols to form dialkyl boronate derivatives. These derivatives are very hydrolytically unstable

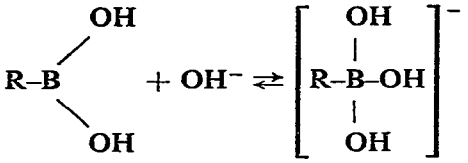


and decompose slowly in moist air. The dialkyl boronate derivatives are often employed to purify crude preparations of boronic acids where use is made of the easy reversibility of the equilibrium reactions; first to form the alkyl boronates which can be distilled and then the original boronic acid is recovered by hydrolysis. The dialkyl boronate derivatives are not usually stable to GC.

The aromatic boronic acids are air stable. The alkylboronic acids (but not their sodium salts) are slowly oxidized by dry air to the orthoboric acid which is moisture sensitive[203,204]. The rate of reaction is influenced by the electron

$$RB(OH)_2 + 1/2\ O_2 \longrightarrow ROB(OH)_2 \xrightarrow{H_2O} ROH + B(OH)_3$$

releasing power of the alkyl substituent. For the butaneboronic acids the stability order is *tert.*-butane < isobutane < *n*-butane. The rate of oxidation is considerably reduced in the presence of moisture and for this reason commercial samples of alkyl boronic acids are supplied as an aqueous slurry and should be stored in a refrigerator. This does not present any problems in their use as derivatizing reagents, as the amount of water required to inhibit the autoxidation reaction is small and for many applications the acid can be used in this form. If it is necessary to remove all the water, then small portions of the acid can be dried on filter paper immediately before use or the slurry can be added to a water scavenger such as 2,2-dimethoxypropane which may be used as the solvent for the derivative reaction. Alternatively the boronic acid can be dissolved in a solvent containing a drying agent (*e.g.*, molecular sieves) which absorbs the water.



Boronic acids behave as acids in the Lewis' sense with the formation of a tetravalent boron atom. Benzeneboronic acid (p*K* 8.86) is three times as strong an acid as boric acid[205] and approximately ten times as strong as *n*-butaneboronic acid[206]. The substitution of electronegative groups into the benzene ring of benzeneboronic acid considerably increases the acid strength whereas alkyl groups decrease the acidity to a much smaller extent. A theoretical interpretation of the effect of substituents on the acid strength of the boronic acids has been given[207,208].

10.2. The formation of boronate derivatives — An overview

The boronate derivatives enjoy widespread use for the analysis of bifunctional compounds by GC. Their dominant position in this area of chromatography is a consequence of their broad range of application, ease of reaction, good thermal and GC properties and their useful mass spectral features. Disadvantages include the poor hydrolytic stability exhibited by many derivatives and the ease of solvolysis observed in multiple derivatization procedures in which the boronate group may be partially or completely displaced when the selective reaction of a remote functional group is required to improve chromatographic properties. Boronate derivatives can be prepared from compounds having two functional groups in close proximity such as alkyl 1,2-diols, 1,3-diols, 1,4-diols, 1,2-enediols, 1,2-hydroxyacids, 1,3-hydroxyacids, 1,2-hydroxyamines, 1,3-hydroxyamines and aromatic compounds with *ortho* substituted phenol, amine and carboxylic acid groups. Many examples of bifunctional

compounds of this type are to be found among the physiologically important classes of compounds such as the steroids, carbohydrates, nucleosides, lipids, catecholamines, prostaglandins etc. Mild conditions are usually sufficient for derivative formation and a typical reaction involves mixing of the boronic acid and substrate in an anhydrous solvent at room temperature for a short time (1.0 min to 0.5 h). In some cases excess boronic acid may be required to force the equilibrium reaction to completion and for those derivatives which are exceptionally moisture sensitive a means of removing water produced in the reaction is required (*e.g.*, molecular sieves can be added to the reaction medium, 2,2-dimethoxypropane can be added as a water scavenger or periodic azeotropic evaporation with benzene or dichloromethane can be used). Direct injection into the gas chromatograph of boronate derivatives with remote unprotected polar functional groups in the presence of excess boronic acid invariable results in poor chromatographic performance exemplified by tailing peaks of reduced peak height. Sequential derivatization of the various functional groups is required in this case and special attention has to be paid to the possibility that strong reaction conditions could result in loss of the boronate group.

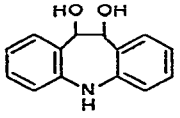
The boronic acids, methaneboronic acid, butaneboronic acid, *tert.*-butaneboronic acid, cyclohexaneboronic acid and benzeneboronic acid have all been used to prepare derivatives for gas chromatography. The cyclohexaneboronates and benzeneboronates have long retention times by comparison to the other boronate derivatives which can be inconvenient for the analysis of poly-bifunctional or high-molecular-weight compounds. The *tert.*-butaneboronates are surprisingly volatile on silicone stationary phases of low polarity with retention times closer to those of the methaneboronates than the *n*-butaneboronates. Unfortunately the reagent and derivatives have poor hydrolytic and air stability which limits their practical use. The methaneboronates are very volatile and the small molecular weight increment formed by derivatization is useful in the MS of high-molecular-weight compounds. The butaneboronate derivatives provide a convenient compromise between volatility and stability making them the most studied derivatives. The stability of the boronate derivatives to TLC and other hydrolysis conditions is variable depending both on local stereochemistry of the bifunctional group and the individual boronic acid used to prepare the derivative.

The boronate derivatives have useful mass spectral properties with prominent molecular ions or quasi [M+1] molecular ions in the case of chemical ionization mass spectrometry. The boronate group is not strongly directing in influencing the mode of fragmentation as charge localization invariably occurs at a center remote from the boronate group due to the electrophilic character of the boron atom. This has the advantage that the abundant ions in the mass spectrum are characteristic of the parent molecule and not the derivatizing reagent. The natural isotope abundance of boron (^{10}B:^{11}B = 1:4.2) aids the identification of boron containing fragments in the low-resolution mass spectra of the boronate derivatives. The boron-isotope distribution is a disadvantage when the mass spectrometer is operated as a single ion gas chromatographic detector, as the ion current carried by the boron containing fragment is divided in the same ratio as the isotope distribution with a consequent reduction in sensitivity.

In the following sections, the usefulness of the boronate derivatives is exemplified by considering their applications under a series of headings representing different types of functional groups and classes of biologically important substances. At the

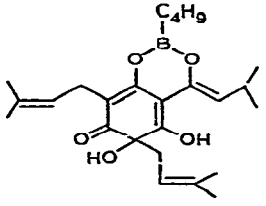
end of each section, all boronate derivatives are collated into a single table for rapid identification of literature sources to particular compounds. The mass spectra of many boronate derivatives have been recorded but as the principal modes of fragmentation observed are more characteristic of the parent molecule than the boronate group *per se*, reference is given to the availability of mass spectral information in the above mentioned tables and is not specifically reviewed here.

10.2.1. Diols and ketols. Diols and ketols react rapidly in solution with boronic acids to form cyclic boronate derivatives with good GC properties. The reaction is specific for *cis*-diols and can be used to distinguish between *cis*- and *trans*-diols by GC with a sample requirement of a few μg[209,210]. Some diols such as 1,3-propanediol and indane-*cis*-1,2-diol form particularly stable boronate derivatives which when added in excess to other boronate diol derivatives result in displacement of the original diol from the derivative which can then be recovered[209,211]. Although the boronate derivatives are generally prepared in solution, they can also be prepared by co-injection of the diol and excess boronic acid into the gas chromatograph in high or quantitative yield. This approach was used to form butaneboronate derivatives for the mass spectral identification of *cis*-diols formed by the metabolism of drugs containing an epoxide ring (*e.g.*, carbamazepine, iminostilbene, cytenamide)[212–215]. The determination of the activity of microsomal styrene monooxygenase (epoxide synthase and epoxide hydrolase using styrene or styrene epoxide as substrate was determined by GC of the phenylethyleneglycol produced after conversion to its butaneboronate[216].



Iminostilbene-10,11-dihydrodiol

Shaw[217] used butaneboronic acid to stabilize the β-hydroxyketol group of the hop bitter acids (humulone, cohumulone, lupulone and colupulone found in beer) and separated the products by GC. The butaneboronate derivatives were said to have useful mass spectral properties for the identification of the isomeric acids. Verzele *et al.*[218] found the butaneboronic and benzeneboronic acid derivatives to be of little value for the separation of this class of compound and recommended that the TMS ethers should be used.

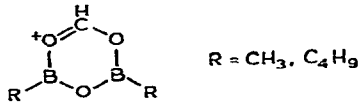


Butaneboronate of Humulone

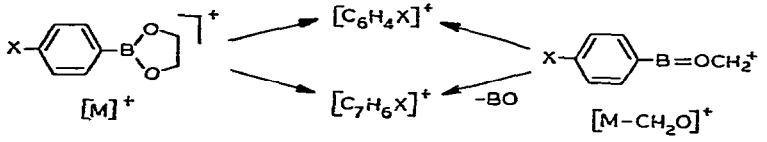
Olefins can be selectively oxidized with osmium tetroxide to the *cis*-diols. Formation of the benzeneboronate and characterization of the derivatives by GC with electron-impact and chemical-ionization MS enabled the original position of the double bond to be established[219]. By capillary column GC, a 35-component mix-

ture of C_5–C_9 monoalkenes was separated after specific oxidation as their benzeneboronate derivatives. The chemical-ionization mass spectra of the benzeneboronates is characterized by a prominent quasi $[M+1]^+$ molecular ion which serves to identify the molecular weight. The electron-impact mass spectra show a characteristic α-cleavage reaction to produce an abundant fragment $[M-R]^+$ identifying the substituent attached to the original double bond.

The methyl esters of mono-unsaturated fatty acids after osmium tetroxide oxidation and formation of the benzeneboronate or butaneboronate derivatives show a similar ion due to α-cleavage[64]. The di-unsaturated and tri-unsaturated fatty acid methyl esters when treated as above did not yield any diagnostically reliable fragmentation ions which could be used to identify the position of the original double bond. The derivatives of the fatty acid methyl esters containing more than one boronate group produced a rearrangement ion containing two boron atoms which was of relatively high abundance but of little diagnostic value.



The origin of the tropylium ion (*m/e* 91) in the mass spectra of the benzeneboronate and *p*-substituted benzeneboronates of 1,2-ethanediol has been fully studied[220–222]. It is known to arise by two independent pathways involving rearrangement under electron-impact conditions. Cyclic hydrocarbon ions containing 7,8,9 and/or 10 carbon atoms are formed in the electron-impact mass spectra of diols with 1,3-, 1,4-etc. substituents[223].



Information concerning the GC of the boronate derivatives of diols and ketols is summarized in Table 15.

10.2.2. Carbohydrates. The boronic acids have been used for several years as a protecting group for *cis*-diols in the synthesis and modification of carbohydrates. This has been reviewed by Ferrier[229]. For synthetic work, benzeneboronates have been preferred over alkaneboronates as they generally give crystalline derivatives with sharp melting points. An acetone solution of benzeneboronic acid in the presence of an acid catalyst gives a moderately high yield of a mixed acetonide–boronate deriva-

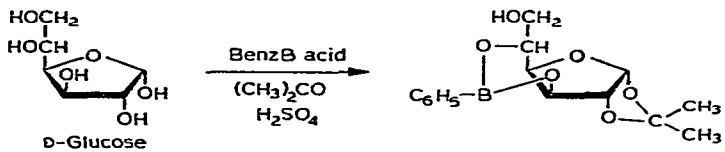
TABLE 15

BORONATE DERIVATIVES USED IN THE GC OF DIOLS AND KETOLS

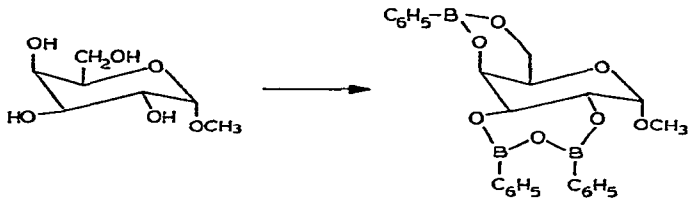
Compound	*Boronate derivative*	*Comments*	*Ref.*
Benzoin	BuB	*I*, EI-MS	211, 224
Colupulone	BuB	GC, EI-MS	217, 218
Cohumulone	BuB	GC, MS	217, 218
Cyclohexane-*cis*-1,2-diol	BuB, BenzB	MU, EI-MS	209
Cycloheximide	BuB	*I*, EI-MS	211, 224
Cytenamide-10,11-dihydrodiol	BuB	GC, EI-MS	214
10,11-Dihydro-10,11-dihydroxy-5H-dibenzo[*a*,*d*]cycloheptene	BuB	GC, EI-MS	213
2,5-Dichloro-2′,3′-biphenyldiol	BuB	EI-MS	225
2,5-Dichloro-3′,4′-biphenyldiol	BuB	EI-MS	225
2,3-Dimethyl-1,2-butanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Dimethyl-2,3-butanediol	BenzB	GC, EI-MS, CI-MS	219
2,6-Dimethyl-1,2-heptanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Dimethyl-2,3-heptanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Dimethyl-2,3-pentanediol	BenzB	GC, EI-MS, CI-MS	219
4,4-Dimethyl-2,3-pentanediol	BenzB	GC, EI-MS, CI-MS	219
Drimane-7ξ,8ξ,11-triol (11 acetate)	BuB, BenzB	MU, EI-MS	209
4,8,13-Duvatriene-1,3-diol	BuB	GC	210
1,2-Ethanediol	BuB	GC	226
2-Ethyl-1,2-hexanediol	BenzB	GC, EI-MS, CI-MS	219
1,2-Heptanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Heptanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Heptanediol	BenzB	GC, EI-MS, CI-MS	219
1,2-Hexanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Hexanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Hexanediol	BenzB	GC, EI-MS, CI-MS	219
2-Hydroxycyclohexanone	BuB	*I*, EI-MS	211, 224
O-(1-Hydroxyethyl) cyclohexanol	BuB	*I*, EI-MS	211, 224
O-Hydroxymethylphenol	BuB	MU, EI-MS	209, 211
Humulone	BuB	GC, EI-MS	217, 218
Iminostilbene-10,11-dihydrodiol	BuB	GC, EI-MS	214
Indane-*cis*-1,2-diol	BuB, BenzB	MU, EI-MS	209
Lupulone	BuB	GC, EI-MS	217, 218
Menthoglycol	BuB	GC, EI-MS	227
2-Methyl-1,2-butanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-2,3-butanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-2,3-heptanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-3,4-hexanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-2,3-pentanediol	BenzB	GC, EI-MS, CI-MS	219
Methyl-*erythro*-9,10-dihydroxystearate (and *threo* isomer)	BuB	*I*, EI-MS	211
Neomenthoglycol	BuB	GC, EI-MS	227
1,2-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
4,5-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
1,2-Octanediol	BenzB	GC, EI-MS, CI-MS	219
1,3-Octanediol	MeB, BuB	GC, EI-MS	228
2,3-Octanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Octanediol	BenzB	GC, EI-MS, CI-MS	219
4,5-Octanediol	BenzB	GC, EI-MS, CI-MS	219

TABLE 15 *(continued)*

Compound	*Boronate derivative*	*Comments*	*Ref.*
1,2-Pentanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Pentanediol	BenzB	GC, EI-MS, CI-MS	219
Phenylethyleneglycol	BuB, BenzB	GC, EI-MS	209
	BuB	GC, EI-MS	216
1,3-Propanediol	BuB	GC	226
Pyridoxine	BuB	*I*, EI-MS	224
2,4,4,-Trimethyl-1,2-pentanediol	BenzB	GC, EI-MS, CI-MS	219



tive with monosaccharides[230]. The boronic acids are generally considered to be specific for *cis*-diol groups in carbohydrates. However, the *trans*-1,2-diol group in methyl α-D-galactopyranoside (also methyl α-D-glucopyranoside) reacts with an excess of benzeneboronic acid in a mixture of dimethylformamide and 2,2-dimethoxypropane (room temperature, 30 min) to form a derivative containing a seven-membered dibenzenepyroboronate ring[231]. The synthesis and characterization by spectroscopy (NMR, EI-MS, OR) was used to confirm the constitution of the benzeneboronate and butaneboronate derivatives of arabinose and xylose[232] and fucose, glucose, fructose and glyceraldehyde[233] which are derivatives used for GC.



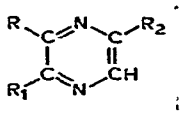
Most of the information concerning the scope of boronic acids to stabilize carbohydrates towards GC has been summarized by Wood and co-workers[234,235] and by Eisenberg[236,237]. The boronic acids are particularly suited to the analysis of monosaccharides with even numbers of substituents in the correct spatial configuration for reaction and to hexoses, hexosamines and uronic acids which can be reduced to alditols, aldosaminitols and aldonolactones respectively. Monosaccharides containing only *trans* orientated hydroxyl groups (*e.g.*, scyllitol, methyl glucoside, sucrose etc.) do not produce peaks on GC[237]. Pyridine is the recommended solvent for reaction and heating is only required if the sugar is insoluble in the solvent. An excess of boronic acid is required to force the reaction to completion[235]. Excess butaneboronic acid (if the derivatives contain no free polar groups) does not present a chromatographic problem as the anhydride formed on injection into the GC elutes with the solvent front under the conditions used for the separation of carbohydrates.

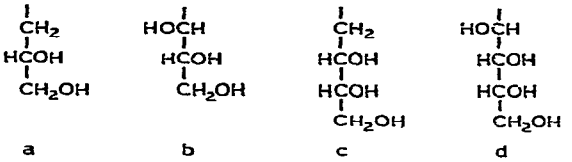
Boronate derivatives of carbohydrates with unprotected functional groups such as hydroxyl, amino and phosphate have poor peak shape and require further derivatization prior to analysis. Phosphate groups are protected by methyl ester formation[238] and hydroxyl and amino groups by trimethylsilylation[234,235,239] or acylation[240]. No satisfactory peaks were observed on GC for the butaneboronate or butaneboronate TMS ether derivatives of D-galactouronic acid, D-glucuronic acid, D-glucurono-6,3-lactone, 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-glucose[235]. The butaneboronates of alditols produced single peaks on GC but arabinitol and xylitol had poor peak shape[235,237]. The formation of TMS ethers with HMDS–TMCS improved the peak shape, but under these conditions, all the alditols (except xylitol) showed the presence of more than one product on GC. The reaction conditions for the formation of the butaneboronate derivatives of fucose, arabinose, xylose and butaneboronate-trimethylsilyl derivatives of fructose, galactose, mannose and glucose have been studied[235]. The boronate-TMS ether derivatives with the exception of the derivative of mannose are stable for at least one weak when stored in the silylation medium. A mixture of BSTFA–TMCS (1:1) was used for the formation of the TMS ethers of the methaneboronate and butaneboronate derivatives of rhamnopyranose, flucopyranose, mannopyranose, glucopyranose, galactopyranose, 2-acetamido-2-deoxy-D-glucopyranose, 2-acetamido-2-deoxy-D-galactopyranose and 2-acetamido-2-deoxy-D-glucopyranose[239]. Acylation of the free hydroxyl groups of the butaneboronate derivatives of glucose, galactose, mannose, fructose and sorbose was complete in one hour at room temperature in a mixture of pyridine and acetic anhydride[240]. The boronate acetate derivatives had good peak shape on GC.

Under carefully controlled conditions, quantitative (or reproducible) yields of cyclic boronates of simple sugars can be obtained and these derivatives are useful in quantitative analysis. The butaneboronate derivatives of glucose and fructose were used for their determination in honey[241], the butaneboronate of 1,2:5,6-dianhydrogalactitol for its determination in plasma[242], the tris(butaneboronate) of sorbitol for its determination in pharmaceutical preparations[243,244] and iduronic and glucoronic acid were determined after conversion to aldonic acids as their tris(butaneboronate) derivatives[245].

The mass spectra of the boronate-TMS carbohydrate derivatives enable the number of carbon atoms (pentose or hexose), the ring size (furanose and pyranose) and the stereochemistry of the hydroxyl groups to be determined[239]. The number of boronate groups identified in the derivative indicates the number of hydroxyl groups in the correct spatial configuration for boronate formation and the number of TMS groups indicates how many further hydroxyl groups are present. In mixed derivatives the boronate group does not strongly direct the mode of fragmentation and this is usually dominated by the TMS[239], acetate[240] or phosphate[238] substituent. The mass spectra of carbohydrate benzeneboronates have been reviewed[229]. Benzeneboronate-TMS ether derivatives of nucleosides have been used for the determination of the base sequence in polynucleosides by mass spectrometry without chromatographic separation[246,247].

The reaction of sugars with ammonia in weakly acid medium results in the formation of a complex mixture of polyhydroxyalkylpyrazines which can be separated by GC of their butaneboronate-TMS ethers and identified by MS[248]. The structures of the nine polyhydroxyalkylpyrazines investigated are given below:





a b c d

R	R_1	R_2
H	CH_3	d
CH_3	H	d
H	a	b
a	H	b
H	b	b
b	b	H
H	c	d
c	H	d
H	d	d

The butaneboronate-TMS derivatives were generally more volatile than the completely trimethylsilylated derivatives on GC but unlike the latter showed some minor peaks in addition to the main peak in some cases.

Those carbohydrates which have been separated by GC of their boronate derivatives are summarized in Table 16.

10.2.3. Steroids. The C-17 side chain of the corticosteroids is thermally labile and eliminated at the temperatures employed for their separation by GC unless protected by the formation of a suitable derivative. The boronic acids have been thoroughly evaluated for this purpose[46,224,250–254]. Methaneboronic acid, butaneboronic acid, *tert.*-butaneboronic acid, cyclohexaneboronic acid and benzeneboronic acid have all been used as derivatizing reagents. The cyclohexaneboronates and benzeneboronates had inconveniently long retention times. The methaneboronates are very volatile with retention times generally less than those of the TMS derivatives. The *tert.*-butaneboronates also have surprisingly short retention times but are very air sensitive and must be handled under nitrogen. The *tert.*-butaneboronic acid reagent was prepared in the hope that the bulky *tert.*-butyl group would enable derivatives to be prepared which were more hydrolytically stable. This was not found to be the case. The butaneboronate derivatives provide a convenient compromise between volatility and hydrolytic stability and have been widely used. Retention index values for a number of corticosteroid boronate derivatives are compiled in Table 17.

Reaction of the corticosteroid side chain with a boronic acid is rapid at room temperature. For 17α,20-diols, 20,21-diols and 17α,20,21-triols, reaction is complete within 5 min when 1 mole equivalent of boronic acid is used[252]. In the case of the 17α, 20,21-triols, reaction most probably occurs at the C-17,21 hydroxyl groups[224]. For derivatization of the 17,21-dihydroxy-20-ketone group a slight excess (1.1 mole equivalents) of boronic acid was required and a reaction time of 15 min for complete reaction. If there are no further free hydroxyl groups in the steroid then a larger excess of boronic acid can be used. Direct injection into the gas chromatograph of a corticosteroid with free hydroxyl groups at C-3, 11 or 20 results in excessive peak tailing and a reduction in peak height for the derivative unless the free hydroxyl group is also protected as its TMS or acetate derivative. Reaction of the C-20,21 ketols even

TABLE 16

CARBOHYDRATES FORMING BORONATE DERIVATIVES USED IN GAS CHROMATOGRAPHY

Compound	*Boronic acid*	*Comments*	*Ref.*
2-Acetamido-2-deoxy-D-galactopyranose	MeB, BuB	GC, EI-MS	239
2-Acetamido-2-deoxy-D-glucopyranose	MeB, BuB	GC, EI-MS	239
2-Acetamido-2-deoxy-D-gulopyranose	MeB, BuB	GC, EI-MS	239
Alditol	BuB	GC	237
Allose	BuB	GC, EI-MS	240
Altose	BuB	GC, EI-MS	240
Arabinitol	BuB	GC	235
Arabinose	BuB	GC	235
	BuB, BenzB	GC	232
	BuB, BenzB	GC, EI-MS	239
	BuB	GC	234
	BuB	GC	237
Ascorbic acid	BuB	GC	237
Chiroinositol	BuB	GC	237
2-Deoxy-D-*erythro*-pentose	BuB	GC	235
1,2:5,6-Dianhydrogalactitol	BuB	GC, EI-MS	242
Erythritol	BuB	GC	234
Fructose	BuB	GC	234
	BuB	GC, EI-MS	235
	BuB	GC, EI-MS	240
	BuB, BenzB	GC, EI-MS	233
Fructose 1-phosphate	BuB, MeB	GC	237
	BuB, MeB	GC, EI-MS	238
Fructose 6-phosphate	BuB, MeB	GC	238
Fucitol	BuB	GC	237
	BuB	GC	234
Fucopyranose	MeB, BuB	GC, EI-MS	239
Fucose	BuB	GC	249
	BuB	GC	237
	BuB, BenzB	GC, EI-MS	233
	BuB	GC	236
	BuB	GC	234
Galactitol	BuB	GC	235
	BuB	GC	237
	BuB	GC	234
Galactopyranose	MeB, BuB	GC, EI-MS	239
Galactose	BuB	GC	234
	MeB, BuB	GC, EI-MS	240
	BuB	GC, EI-MS	235
	BuB	GC, EI-MS	237
Galactose 1-phosphate	MeB, BuB	GC, EI-MS	238
Galactose 6-phosphate	MeB, BuB	GC	237
Galactoseaminitol	BuB	GC	237
Gluconolactone	BuB	GC	237
Glucopyranose	MeB, BuB	GC, EI-MS	239
Glucitol	BuB	GC	237
	BuB	GC	235
	BuB	GC	234
	MeB, BuB	GC	234
Glucoronic acid 6-phosphate	MeB, BuB	GC, EI-MS	245

TABLE 16 *(continued)*

Compound	*Boronic acid*	*Comments*	*Ref.*
Glucose	BuB	GC	234
	MeB, BuB	GC, EI-MS	240
	BuB, BenzB	GC, EI-MS	233
	BuB, BenzB	GC	237
	BuB, BenzB	GC, EI-MS	237
Glucoronic acid	BuB, BenzB	GC, EI-MS	245
Glucose 6-phosphate	BuB	GC, EI-MS	235
Glucose aminitol	BuB	GC	235
Glucoronic acid	BuB	GC	245
Glyceraldehyde	BuB, BenzB	GC, EI-MS	233
Guloiactone	BuB	GC	237
Iditol	BuB	GC	237
Idonolactone	BuB	GC	237
Idose	BuB	GC, EI-MS	240
Iduronic acid	BuB	GC	245
Lyxose	BuB	GC	234
Methyl-D-Mannopyranose	MeB, BuB, BenzB	GC, EI-MS	239
Mannitol	BuB	GC	234
	BuB	GC	244
	BuB	GC	245
Sorbitol	BuB	GC	243
	BuB	GC	244
Sorbose	BuB	GC	240
Tagatose	BuB	GC	240
Talose	BuB	GC	240
Xylitol	BuB	GC	234
	BuB	GC	235
	BuB	GC	237
Xylose	BuB	GC	234
	BuB, BenzB	GC	249
	BuB, BenzB	GC, EI-MS	232
	BuB	GC	235
	BuB	GC	237

with excess boronic acid does not proceed to completion and there is evidence that partial decomposition of the derivative may also occur on GC. The boronate derivatives of 17,20- and 20,21-diols and 17,20,21-triols are stable to both BSA and HMDS–TMCS when used to form TMS derivatives of unprotected hydroxyl groups and to acetic anhydride-pyridine used to form acetate derivatives[46,224,252]. Under similar conditions the 17,21-dihydroxy-20-ketone and 20, 21-ketal boronates gave a mixture of products with loss of the boronate group. Methyloxime derivatives of ketones can be formed prior to or after boronate formation without problems. The boronate derivatives of the 17,20- and 20,21-diols and 17,20,21-triols yielded stable derivatives which resisted hydrolysis and could be submitted to TLC. The boronate ester derivative can be displaced from the corticosteroid side chain by addition of an excess of 1,3-propanediol for recovery of the original steroid[224,250–252].

An interesting cyclic derivative of 18-hydroxy-11-deoxycorticosterone is formed by reaction with methaneboronic acid involving a molecular re-arrangement in the corticosteroid side chain[254].

TABLE 17

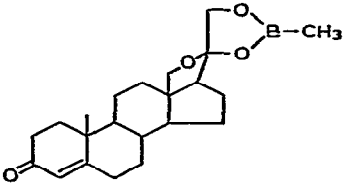
RETENTION INDEX VALUES OR METHYLENE UNITS FOR CORTICOSTEROID BORONATES OBTAINED BY GC ON OV-17

6 ft. × 4 mm I.D. glass column of 1% OV-17 on Gas-Chrom Q, 100–120 mesh. MU values determined by temperature programming from 230° at 2°/min.

Corticosteroid	*Boronic acid*	*Retention index for derivative*	*Column temperature (°C)*
5β-Pregnane-3α,17α,20α-triol	MeB	3010	240
	t-BuB	3050	240
	BuB	3265	MU
	CHB	3590	250
	BenzB	3775	250
5β-Pregnane-3α,17α,20β-triol	MeB	2970	240
	t-BuB	3010	240
	BuB	3265	MU
	BenzB	3775	250
5β-Pregnane-3α,11β,17α,20β-tetrol	MeB	3255	250
	t-BuB	3270	250
	BuB	3480	MU
20β,21-Dihydroxypregn-4-ene-3-one	MeB	3380	250
	t-BuB	3520	250
	BuB	3680	MU
	CHB	4030	250
	BenzB	4330	250
5α-Pregnane-3α,11β,20α,21-tetrol	MeB	3460	250
	t-BuB	3600	250
5α-Pregnane-3α,11β,20β,21-tetrol	MeB	3470	250
	t-BuB	3565	240
17α,20α,21-Pregnan-4-ene-3-one	MeB	3595	250
	t-BuB	3650	250
	CHB	4205	250
17α,20β,21-Trihydroxy-pregnan-4-ene-3-one	MeB	3620	250
	t-BuB	3650	250
	BuB	3835	250
	CHB	4080	250
	BenzB	4345	250
5α-Pregnane-3β,11β,17α,20β,21-pentol	MeB	3650	250
	t-BuB	3715	250
3α,17α,20α,21-Tetrahydroxy-5β-pregnane-11-one	MeB	3490	250
	t-BuB	3570	250
	BuB	3800	MU
17α,21-Dihydroxypregnan-4-ene-3,20-dione	MeB	3360	250
	t-BuB	3400	250
	BuB	3580	MU
	CHB	3965	250
	BenzB	4120	250
17α,21-Dihydroxy-5β-pregnane-3,20-dione	MeB	3175	240
	t-BuB	3215	240
	BuB	3400	MU
	CHB	3710	250
	BenzB	3885	250
17α,21-Dihydroxypregnan-4-ene-3,11,20-trione	MeB	3450	250
3α,17α,21-Trihydroxy-5β-pregnane-20-one	MeB	3180	250
	t-BuB	3220	250
	BuB	3345	MU

TABLE 17 *(continued)*

Corticosteroid	*Boronic acid*	*Retention index for derivative*	*Column temperature (°C)*
	t-BuB	3490	250
	BuB	3660	MU
	CHB	4065	250
	BenzB	4230	250
17α,21-Dihydroxy-5β-pregnane-3,11,20-trione	MeB	3300	250
	t-BuB	3305	250
	BuB	3660	MU
	CHB	3825	250
	BenzB	3990	250
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	MeB	3270	250
	t-BuB	3270	240
	BuB	3465	MU
11β,17α,21-Trihydroxypregnan-4-ene-3,20-dione	MeB	3630	250
	t-BuB	3660	250
	BuB	3890	250
3α,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one	MeB	3360	240
	t-BuB	3400	240
	BuB	3605	MU
21-Hydroxypregnan-4-ene-3,20-dione	MeB	3310	250
	t-BuB	3480	250
21-Hydroxy-5β-pregnane-3,20-dione	MeB	3220	250
	t-BuB	3380	250
3β-21-Dihydroxy-pregnane-5-ene-20-one	MeB	3150	250
	t-BuB	3325	MU
	BuB	3470	250
	BenzB	3850	250



Methaneboronate of 18-hydroxy-11-deoxycorticosterone

The mass spectra of the corticosteroid boronates are characterized by a prominent molecular ion fragmenting to produce abundant daughter ions characteristic of the parent steroid[253]. In most cases the positive charge is localized on the steroid nucleus yielding diagnostically informative mass spectra. The chemical-ionization mass spectra of the methaneboronate derivative of 18-hydroxy-11-deoxycorticosteroid has the quasi $[M+1]^+$ molecular ion as its base peak with very little further fragmentation[254].

Steroids which have been separated by GC as their boronate derivatives are summarized in Table 18.

10.2.4. Prostaglandins. The ability to form a boronate derivative has been used to distinguish the prostaglandin F series from the E series. Only the F prostaglandins have a 9α,11α *cis*-diol group, which is the site of boronate formation. The boronic acids react specifically with the *cis*-diol group as the prostaglandin F_β series

TABLE 18

BORONATE DERIVATIVES FOR THE GAS CHROMATOGRAPHIC SEPARATION OF STEROIDS

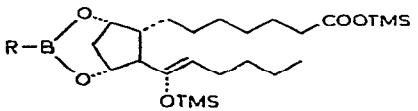
Steroid	*Boronate derivative*	*Comments*	*Ref.*
Androstanes			
3β,16α,17α-Trihydroxyandrost-5-ene	BuB	MU, EI-MS	250
3β,16β,17β-Trihydroxyandrost-5-ene	BuB	MU, EI-MS	250
Cholestanes			
2β,3β-Dihydroxy-5α-cholestane	BuB	GC	255
Pregnanes			
21-Hydroxy-5β-pregnane-3,20-dione	BuB	MU, EI-MS	252
21-Hydroxy-5α-pregnane-3,20-dione	MeB, t-BuB	*I*, EI-MS	252
21-Hydroxypregnan-4-ene-3,20-dione	MeB, t-BuB	*I*, EI-MS	252
18-Hydroxy-11-deoxycorticorsteroid	MeB	*I*, EI-MS, CI-MS	254
3β,21-Dihydroxypregnan-5-ene-20-one	MeB, BuB, t-BuB		252
	BenzB	*I*, EI-MS	
	BuB	MU, EI-MS	250
17α,21-Dihydroxy-5β-pregnane-3,20-dione	MeB, BuB, t-BuB		
	CHB, BenzB	*I*, EI-MS	250
	BuB	MU, EI-MS	250
17α,21-Dihydroxypregnan-4-ene-3,20-dione	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB		252
	CHB, BenzB	*I*, EI-MS	
	MeB	*I*, EI-MS	46
17α,21-Dihydroxy-5β-pregnane-3,11,20-trione	MeB, BuB, t-BuB		
	CHB, BenzB	*I*, EI-MS	252
	BuB	MU, EI-MS	250
17α,21-Dihydroxypregnan-4-ene-3,11,20-trione	MeB, BuB, t-BuB		
	CHB, BenzB	*I*, EI-MS	252
	MeB, BuB		
	t-BuB, BenzB	*I*, EI-MS	251
	BuB	MU, EI-MS	250
20β,21-Dihydroxypregn-4-ene-3-one	MeB, BuB, t-BuB		
	CHB, BenzB	*I*, EI-MS	252
	MeB	*I*, EI-MS	46
3α,17α,20α-Trihydroxy-5β-pregnane	BuB, BenzB	MU, EI-MS	209
	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB		
	CHB, BenzB	MU, EI-MS	251
-3-TMS-ether	BuB	MU, EI-MS	250
-3-acetate ester	BuB	MU, EI-MS	250
3α,17α,20β-Trihydroxy-5β-pregnane	MeB	*I*, EI-MS	46
	MeB, BuB, t-BuB		
	CHB, BenzB	*I*, EI-MS	251
3β,17α,20α-Trihydroxypregn-5-ene	BuB	MU, EI-MS	250
3β,17α,20β-Trihydroxypregn-5-ene	BuB	MU, EI-MS	250
3α,17α,21-Trihydroxy-5β-pregnane-3-one	MeB, BuB, t-BuB	MU, EI-MS	252
3α,20α,21-Trihydroxy-5β-pregnane-11-one	BuB	MU, EI-MS	250
3α,17α,21-Trihydroxy-5β-pregnane-20-one	BuB	MU, EI-MS	250
	BuB	MU, EI-MS	252
	BuB	GC, EI-MS	224
3α,17α,21-Trihydroxypregnane-11,20-dione	BuB	MU, EI-MS	252
	MeB, BuB, t-BuB	*I*, EI-MS	250
17α,20α,21-Trihydroxypregn-4-ene-3-one	BuB	GC, EI-MS	224

TABLE 18 *(continued)*

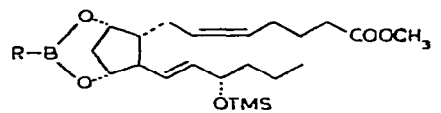
Steroid	*Boronate derivative*	*Comments*	*Ref.*
	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB		252
	CHB	*I*, EI-MS	252
17α,20β,21-Trihydroxypregn-4-ene-3-one	MeB	*I*, EI-MS	46
	MeB, BuB, t-BuB		
	CHB, BenzB	*I*, EI-MS	252
	BuB	GC, EI-MS	224
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB	*I*, EI-MS	252
3α,11β,17α,20β-Tetrahydroxy-5β-pregnane	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB	*I*, EI-MS	252
3α,11β,20α,21-Tetrahydroxy-5α-pregnane	MeB, t-BuB	MU, EI-MS	252
3α,11β,20α,21-Tetrahydroxy-5α-pregnane	t-BuB	MU, EI-MS	250
3α,11β,20β,21-Tetrahydroxy-5α-pregnane	MeB, t-BuB	MU, EI-MS	250
3α,11β,20β,21-Tetrahydroxy-5β-pregnane	t-BuB	MU, EI-MS	250
3α,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB	MU, EI-MS	252
3α,17α,20α,21-Tetrahydroxy-5β-pregnane-11-one	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB	*I*, EI-MS	252
3α,17α,20β,21-Tetrahydroxy-5β-pregnane-11-one	BuB	MU, EI-MS	250
3β,11β,17α,20β,21-Pentahydroxy-5β-pregnane	MeB, t-BuB	MU, EI-MS	252

which contain a *trans*-diol group do not form a derivative[256]. The selectivity of the reaction for the *cis*-diol group has been important in identifying the presence of the prostaglandin F_α series in biological samples (sheep blood[257], human semen[258], human aortal media[259], rat stomach homogenate[260], and rat uterus media[261]) when only small quantities of extracts were available for study.

The prostaglandins are multifunctional compounds and mixed derivatives are prepared for their GC analysis. Either the cyclic boronate TMS ether TMS ester derivative[256] or the cyclic boronate TMS ether methyl ester derivative were prepared[257–265]. A disadvantage of the cyclic boronate TMS ether TMS ester derivatives was their ease of solvolysis. The butaneboronate derivative of prostaglandin $F_{1\alpha}$ is slowly converted to the fully trimethylsilylated derivative beginning after about 2 h at room temperature in a solution of Trisil Z[256]. In a 5% solution of BSTFA in hexane, the butaneboronate TMS ether methyl ester derivative of prostaglandin $F_{2\alpha}$ was stable



Prostaglandin $F_{1\alpha}$ cyclic boronate TMS ether TMS ester derivative



Prostaglandin $F_{2\alpha}$ cyclic boronate TMS ether methyl ester derivative

at room temperature for up to ten days and considerably longer at −15° (ref. 263). Brief mention has been made of the formation of a 19-hydroxyprostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ butaneboronate *tert.*-butyldimethylsilyl ether methyl ester derivative which have as their base peak, the ion $[M-tert.\text{-}Bu]^+$ in the mass spectrum .Various boronic acids have been evaluated for the separation of prostaglandin $F_{1\alpha}$, $F_{2\alpha}$ and $F_{3\alpha}$ as their cyclic boronate TMS ether methyl ester derivatives (Table 19)[265]. The prostaglandin $F_{2\alpha}$ and $F_{3\alpha}$ derivatives are not separated adequately on packed or capillary columns of OV-1 or OV-17. Chemical-ionization MS can be used to distinguish between the different F_α prostaglandins by identification of the base peak $[M-RBO_2H_2-TMSO]^+$ in the mass spectrum which occurs at a different *m/e* value for each prostaglandin[265]. In the electron-impact mass spectra the base peak $[M-71]^+$ of the prostaglandin $F_{2\alpha}$ derivative occurs at the same *m/e* value (393 a.m.u.) as the base peak of the prostaglandin $F_{3\alpha}$ $[M-69]^+$ derivative. This ion is often chosen for the determination of prostaglandin $F_{2\alpha}$ by single ion monitoring[259,263,264]. As the prostaglandin $F_{2\alpha}$ and $F_{3\alpha}$ derivatives are not separated chromatographically some problems may arise in confirming the identities of these two prostaglandins.

TABLE 19

RETENTION INDEX VALUES FOR THE CYCLIC BORONATE TMS ETHER METHYL ESTER DERIVATIVES OF PROSTAGLANDINS $F_{1\alpha}$, $F_{2\alpha}$ AND $F_{3\alpha}$ ON OV-1

2 m × 4 mm I.D. glass column of 1% OV-1 on Gas-Chrom Q (100–120 mesh) at 225° isothermally with a nitrogen flow-rate of 50 ml min^{-1}.

Boronate derivative	*Prostaglandin*	*Retention index*
Methaneboronate	$F_{1\alpha}$	2625
	$F_{2\alpha}$	2585
	$F_{3\alpha}$	2590
Butaneboronate	$F_{1\alpha}$	2885
	$F_{2\alpha}$	2850
	$F_{3\alpha}$	2845
Cyclohexaneboronate	$F_{1\alpha}$	3160
	$F_{2\alpha}$	3125
	$F_{3\alpha}$	3120
Benzeneboronate	$F_{1\alpha}$	3230
	$F_{2\alpha}$	3185
	$F_{3\alpha}$	3180

The prostaglandin F_α series which have been separated by GC of their boronate derivatives are summarized in Table 20.

10.2.5. Lipids. Sphingosines, ceramides, monoglycerides and monoalkyl glyceryl ethers can be determined by GC after formation of their cyclic boronate derivatives[254,266–269]. The sphingosines are sphingolipid long-chain bases (sphinganine, D-*erythro*-1,3-dihydroxy-2-aminooctadecane; 4-sphingenine, D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene; 4-D-hydroxysphinganine, D-ribo-1,3,4-trihydroxy-2-aminooctadecane) and are readily converted to their boronate esters by reaction with a slight excess of boronic acid (1.1 mole equivalent) in pyridine at room temperature for 10 min[254,266]. MS indicates that boronate formation takes place at the 1,3-diol group of the 2-amino-1,3-diol side chain and that in the case of 4-D-hydroxy-

TABLE 20

BORONATE DERIVATIVES FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF PROSTAGLANDINS

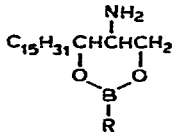
Prostaglandin	*Boronate derivative*	*Comments*	*Ref.*
Prostaglandin $F_{1\alpha}$	BuB	GC, EI-MS	256
	MeB, BuB, CHB		
	BenzB	*I*, EI-MS, CI-MS	265
	BuB	GC, EI-MS	262
	BuB	GC, EI-MS	263
	BuB	GC, EI-MS	264
Prostaglandin $F_{2\alpha}$	BuB	GC, EI-MS	256
	MeB, BuB, CHB		
	BenzB	*I*, EI-MS, CI-MS	265
	MeB, BuB, CHB		
	BenzB	*I*, EI-MS	259
	BuB	GC, EI-MS	263
	BuB	GC, EI-MS	257
Prostaglandin $F_{3\alpha}$	MeB, BuB, CHB		
	BenzB	*I*, EI-MS, CI-MS	265
19-Hydroxyprostaglandin $F_{1\alpha}$	BuB	GC, EI-MS	258
19-Hydroxyprostaglandin $F_{2\alpha}$	BuB	GC, EI-MS	258
6-Ketoprostaglandin $F_{1\alpha}$	BuB	GC, EI-MS	261
	BuB	GC, EI-MS	260

TABLE 21

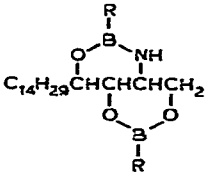
RETENTION INDICES FOR SPHINGOSINE BORONATE DERIVATIVES ON GAS CHROMATOGRAPHY

2 m × 3 mm I.D. glass column of 1% stationary phase on Gas-Chrom Q (100–120 mesh). Flow-rate 50 ml min^{-1}. DMAM = N,N-dimethylaminomethylene.

Compound	*Derivative*	*Column temperature (°C)*	*Stationary phase*	
			OV-1	*OV-17*
Sphinganine	MeB	200	2315	2480
	BuB	230	2590	2740
	BenzB	230	2940	3220
	MeB, DMAM	250	2570	2765
	BuB, DMAM	230	2840	3020
	BenzB, DMAM	270	3260	3535
	N-Acetyl, MeB	230	2600	2835
	N-Acetyl, BuB	270	2850	3080
	N-Acetyl, BenzB	270	3210	3595
	Acetone Schiff base, MeB	230	2420	2565
	Acetone Schiff base, BuB	230	2675	2815
	Acetone Schiff base, BenzB	270	3040	3310
4-Sphingenine	MeB	200	2305	2485
	BenzB	230	2940	3270
	Acetone Schiff base, MeB	230	2420	2580
	Acetone Schiff base, BenzB	270	3030	3360
4 D-Hydroxy-sphinganine	Bis-MeB	230	2425	2570

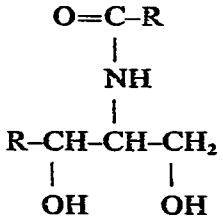


Cyclic Boronate Derivative of Sphinganine



Bis(boronate) Derivative of 4-D-hydroxysphinganine

sphinganine a bis(boronate) derivative was formed. The sphinganine boronate and the bis(boronate) derivative of 4-D-hydroxysphinganine had poor chromatographic properties unless the amine group was also derivatized. The boronate derivative was stable to the subsequent conversion of the amine group to an N-acetyl, N,N-dimethylaminomethylene or Schiff base derivative. The Schiff base derivatives had the shortest retention times and superior peak shape on GC. Retention index data for some derivatives of sphingosine are given in Table 21. All derivatives with the exception of the N,N-dimethylaminomethylene derivatives could be stored for several days at room temperature without decomposition and even longer in a refrigerator[254,266,269].



General Structure of Ceramides

Ceramides are N-acyl sphingosines containing a 2-amido-1,3-diol structure. They form cyclic boronate derivatives via the 1,3-diol group. The methaneboronate derivatives were formed by adding a 1.1 molar excess of boronic acid (2.2 molar in the case of ceramides derived from 2-hydroxyacids) to the ceramide in pyridine and allowing the reaction to proceed for 10 min at room temperature[267]. For ceramides derived from the enzymatic hydrolysis of lysolecithin and sphingomyelin a 1.5 mole excess of boronic acid was reacted with the ceramide in ethyl acetate solution for 30 min at room temperature[268]. Retention index data for some ceramide methaneboronate derivatives is given in Table 22. The derivatives were stable in solution for several days at room temperature, could be submitted to TLC without decomposition and were stable to treatment with pyridine–HMDS–TMS (5:4:3) at room temperature for 30 min.

Lipids which have been determined by GC after formation of their boronate derivatives are summarized in Table 23.

10.2.6. Hydroxyamines and hydroxyacids. The butaneboronate derivatives of hydroxyamines and hydroxyacids have good peak shape and in the case of the hydroxyacids, longer retention times by 300–400 retention index units compared to the methyl hydroxyesters on OV-17 (ref. 211). Boronate derivatives can be formed with α and β substituted hydroxyacids and amines at room temperature in an anhydrous

TABLE 22

RETENTION INDICES FOR CERAMIDE AND MONOALKYL GLYCERIDE METHANEBORONATES ON OV-1 AS STATIONARY PHASE

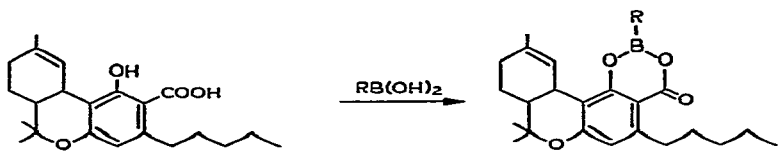
Compound	*Retention index*
*Ceramides**	
N-Myristoyl sphinganine	3702
N-Palmitoyl sphinganine	3896
N-Palmitoleoyl sphinganine	3886
N-Stearoyl sphinganine	4097
N-Oleoyl sphinganine	4073
N-Linoleoyl sphinganine	4074
N-Palmitoyl 4-sphingenine	3912
N-Palmitoleoyl 4-sphingenine	3898
*Monoalkyl glycerides***	
Glyceryl 1-hexadecenoate	2355
Glyceryl 1-hexadecanoate	2373
Glyceryl 1-heptadecanoate	2478
Glyceryl 1-octadecenoate	2552
Glyceryl 1-octadecanoate	2572

* 40 m × 0.55 mm I.D. capillary column, pretreated with silanox, temperature 300°, helium flow-rate 15 ml min^{-1}.

** 30 m × 0.5 mm I.D. capillary column, pretreated with silanox, temperature 230°.

solvent. The derivatives are stable to storage for at least 2–3 days in an anhydrous solvent and several derivatives were stable to TLC[211]. Retention index values for some representative hydroxyamines and hydroxyacids are summarized in Table 24.

The salicylate group in cannabinolic acids was converted to its boronate derivative by adding an excess of boronic acid to the sample dissolved in pyridine-benzene (1:1). After 30 min at room temperature, the solvent was evaporated under nitrogen and the procedure repeated again[270]. A double derivatization technique was essential to remove water and force the reaction to completion. The boronate derivatives were stable to treatment with CH_3CN–BSTFA–TMCS (2:2:1) at room temperature for thirty minutes and could be stored for several weeks at 4° without decomposition. The benzeneboronate derivatives like the TMS derivatives were not thermally stable with some decomposition occurring on-column. The methaneboronate and butaneboronate derivatives were thermally stable and had good peak shape. The methaneboronate derivatives had comparable retention times to those of the TMS derivatives, whereas the butaneboronates had longer retention times and methylene unit values about three units higher. The butaneboronate derivatives enabled a complete separation of the cannabinoids from their acids to be achieved on a single packed column.



Δ¹-Tetrahydrocannabinolic acid

TABLE 23

BORONATE DERIVATIVES USED IN THE GAS CHROMATOGRAPHY OF LIPIDS

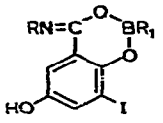
Compound	*Boronic acid*	*Comments*	*Ref.*
Hydroxyfatty acid alkyl esters			
4,5-Dihydroxydocosanoate	BuB, MeB	GC, EI-MS	64
8,9-Dihydroxydodecanoate	BuB, MeB	GC, EI-MS	64
9,10-Dihydroxyoctadecanoate	BuB, MeB	GC, EI-MS	64
5,6,8,9,11,12-Hexahydroxydodecanpropionate	BuB, MeB	GC, EI-MS	64
9,10,12,13,15,16-Hexahydroxyoctadecanpropionate	BuB, MeB	GC, EI-MS	64
4,5,7,8-Tetrahydroxydocosanethanoate	MeB	GC, EI-MS	64
9,10,12,13-Tetrahydroxyoctadecanethanoate	BuB, MeB	GC, EI-MS	64
Glyceryl ethers			
1-Hexadecyl glyceryl ether	MeB	*I*, EI-MS, CI-MS	254
2-Hexadecyl glyceryl ether	MeB	*I*, EI-MS, CI-MS	254
Glyceryl alkyl esters			
Glyceryl 1-heptadecanoate	MeB	*I*, EI-MS	268
Glyceryl 1-myristate	BuB, BenzB	MU, EI-MS	209
Glyceryl 1-octadecenoate	MeB	*I*, EI-MS	268
Glyceryl 1-palmitate	MeB	*I*, EI-MS, CI-MS	254
	MeB	*I*, EI-MS	268
Glyceryl 1-palmitoleate	MeB	*I*, EI-MS	68
Glyceryl 1-stearate	MeB	*I*, EI-MS	58
	MeB	*I*, EI-MS, CI-MS	254
Sphingosines			
Dihydrosphingosine	MeB	*I*, EI-MS, CI-MS	64
N-Ethyl sphinganine	MeB, BuB, BenzB	GC, EI-MS, CI-MS	228
N-Ethyl 4-sphingenine	MeB, BuB, BenzB	GC, EI-MS, CI-MS	228
4D-Hydroxysphinganine	MeB, BuB, BenzB	GC, EI-MS, CI-MS	228
	MeB, BuB, BenzB	*I*, EI-MS, CI-MS	266
4-Sphingenine	MeB, BuB, BenzB	*I*, EI-MS, CI-MS	266
Ceramides			
N-Behenoyl-4-sphingenine	MeB	*I*, EI-MS	268
N-2-Hydroxystearoyl-4-sphingenine	MeB	*I*, EI-MS	267
N-Lignoceronyl-4-sphingenine	MeB	*I*, EI-MS	268
N-Linoleoyl sphinganine	MeB	*I*, EI-MS	267
N-Myristoyl sphinganine	MeB	*I*, EI-MS	267
N-Myristoyl-4-sphingenine	MeB	*I*, EI-MS	267
N-Nervonoyl-4-sphingenine	MeB	*I*, EI-MS	268
N-Oleoyl sphinganine	MeB	*I*, EI-MS	267
N-Palmitoleoyl sphinganine	MeB	*I*, EI-MS	267
N-Palmitoleoyl-4-sphingenine	MeB	*I*, EI-MS	267
	MeB	*I*, EI-MS	267
N-Palmitoyl-D,L-dihydrosphingosine	MeB	*I*, EI-MS	254
N-Palmitoyl-4-heptadecasphingenine	MeB	*I*, EI-MS	267
N-Palmitoyl-4-hexadecasphingenine	MeB	*I*, EI-MS	267
N-Palmitoyl-4-sphingenine	MeB	*I*, EI-MS	268
	MeB	*I*, EI-MS	267
N-Stearoyl-D,L-dihydrosphingosine	MeB	*I*, EI-MS	254
N-Stearoyl-4-dodecasphingenine	MeB	*I*, EI-MS	268
N-Stearoyl-4-hexadecenylsphingenine	MeB	*I*, EI-MS	268
N-Stearoyl sphinganine	MeB	*I*, EI-MS	267
N-Stearoyl-4-sphingenine	MeB	*I*, EI-MS	268

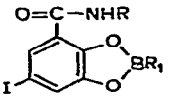
TABLE 24

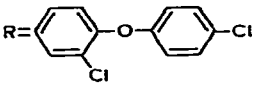
RETENTION INDEX VALUES FOR SOME BUTANEBORONATE DERIVATIVES OF HYDROXYACIDS AND HYDROXYAMINES ON OV-17

Column temperature not given. 6 ft. × $^1/_8$ in. I.D. glass column of 1% OV-17 on Gas-Chrom Q.

Compound	*Retention index*
α-Hydroxyacids	
Lactic Acid	1200
α-Hydroxyisobutyric acid	1170
α-Hydroxymyristic acid	2245
Mandelic acid	1805
Tartaric Acid	1940
β-Hydroxyacids	
3-Hydroxypropionic acid	1420
3-Hydroxybutyric acid	1410
cis-2-Hydroxycyclohexanecarboxylic acid	1747
trans-2-Hydroxycyclohexanecarboxylic acid	1768
Salicylic Acid	1792
3-Methoxysalicylic acid	2110
4-Methoxysalicylic acid	2145
6-Methoxysalicylic acid	2180
Thymotic acid	2035
2-Hydroxy-1-naphthoic acid	2450
2-Hydroxy-3-naphthoic acid	2480
Hydroxyamines	
o-Aminophenol	1670
o-Phenylenediamine	1820
3-Aminopropanol	1218

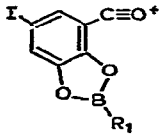






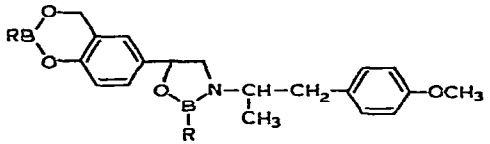
$R_1 = CH_3 . C_4H_9$

The butaneboronate and benzeneboronate derivatives were used to distinguish between the two isomeric hydroxy-salicylanilides, 3′-chloro-4′-(*p*-chlorophenoxy)-3-hydroxy-5-iodosalicylanilide and 3′-chloro-4′-(*p*-chlorophenoxy)-5-hydroxy-3-iodosalicylanilide by MS [271]. The two isomers formed different types of boronate derivatives which had clearly different mass spectra. The diol derivative had as its base peak the ion *m/e* (272 + R_1) which was absent in the mass spectra of the hydroxyamide derivative.



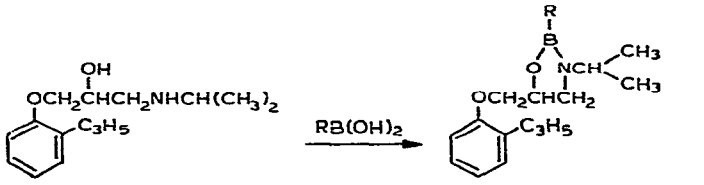
m/e (272 + R_1)

Salmefamol and labetalol (substituted propanolamines) form a bis(boronate) derivative when reacted with excess butaneboronic or benzeneboronic acid in anhydrous dimethylformamide for twenty minutes at room temperature[58]. The boronate derivatives gave a good separation of the possible diastereoisomeric forms by GC but the quantitative results were found to be unreliable due to on-column decomposition.



Cyclic Boronate Derivative of Salmefamol

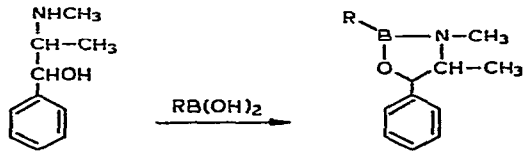
Alprenolol contains a propanolisopropylamine side chain which can be converted to its butaneboronate derivative by either heating at 80° for 2 h with excess butaneboronic acid[272] or by co-injection with a solution of butaneboronic acid in dimethylformamide[193,273]. Butaneboronate formation used in conjunction with GC–EI-MS was useful for the identification of the drug and its metabolites in biological fluids, as several of the metabolites formed either retained the side chain composition intact or contained other bifunctional groups as a result of enzymatic modifications.



Alprenolol Alprenolol Butaneboronate

Hydroxyamines and hydroxyacids which have been separated as their boronate derivatives by GC are summarized in Table 25.

10.2.7. Catecholamines. The catecholamines can be derivatized by a 1.5–2.0 mole excess of boronic acid in pyridine or dimethylformamide overnight at room temperature[224,274]. Acetone is unsuitable as a solvent for the reaction due to the formation of secondary products, identified as oxazolidines in the case of secondary β-hydroxyamines. Yields were not always quantitative under these conditions[274] and catecholamines with remaining unblocked polar groups (*e.g.*, synephrine, neosynephrine, octopamine etc.) had unsatisfactory chromatographic properties. However, attempted trimethylsilylation of unprotected phenol groups resulted in cleavage of the butaneboronate from the β-hydroxyamine function. The formation of boronate derivatives enabled a separation of the diastereoisomeric pair ephedrine and pseudo-



(61)

Ephidrine Ephidrine Boronate Derivative

TABLE 25

BORONATE DERIVATIVES USED FOR THE GAS CHROMATOGRAPHY OF HYDROXYAMINES AND HYDROXYACIDS

Compound	*Boronate derivative*	*Comments*	*Ref.*
Allothreonine methyl ester	BuB	*I*, EI-MS	211
Alprenolol (and metabolites)	BuB	GC, EI-MS	193
			272
			273
2-Amino-2-methylpropanol	BuB	*I*, EI-MS	211
o-Aminophenol	BuB	*I*, EI-MS	211
2-Aminopropanol	BuB	*I*, EI-MS	211
3-Aminepropanol	BuB	*I*, EI-MS	211
	BuB	*I*, EI-MS	224
Cannabidiolic acid	MeB, BuB, BenzB	*I*, EI-MS	270
Cannabinolic acid	MeB, BuB, BenzB	*I*, EI-MS	270
Ethanolamine	BuB	*I*, EI-MS	211
3-Hydroxybutyric acid	BuB	*I*, EI-MS	211
cis+*trans*-2-Hydroxycyclohexanecarboxylic acid	BuB	*I*, EI-MS	211
	BuB	*I*, EI-MS	224
2-Hydroxyisobutyric acid	BuB	*I*, EI-MS	211
4-Hydroxy-3-methyoxymandelic acid	BuB	*I*, EI-MS	211
2-Hydroxymyristic acid	BuB	*I*, EI-MS	211
2-Hydroxy-1-naphthoic acid	BuB	*I*, EI-MS	211
2-Hydroxy-3-naphthoic acid	BuB	*I*, EI-MS	211
3-Hydroxypropionic acid	BuB	*I*, EI-MS	211
Labetalol	BuB, BenzB	GC	58
Lactic acid	BuB	*I*, EI-MS	224
	BuB	*I*, EI-MS	211
Mandelic acid	MeB, BuB, BenzB, CHB	*I*, EI-MS	211
	BuB	*I*, EI-MS	224
3-Methoxysalicylic acid	BuB	*I*, EI-MS	211
4-Methoxysalicylic acid	BuB	*I*, EI-MS	211
6-Methoxysalicylic acid	BuB	*I*, EI-MS	211
Phenylpropanolamine	MeB, BuB, BenzB, CHB	*I*, EI-MS	274
Phenylpyruvic acid	BuB	*I*, EI-MS	211
	BuB	*I*, EI-MS	224
Propylcannabinolic acid	MeB, BuB	*I*, EI-MS	270
Propyl-Δ'-tetrahydrocannabinolic acid	MeB, BuB	*I*, EI-MS	270
Salicylic acid	BuB	*I*, EI-MS	224
	MeB, BuB, BenzB, CHB	*I*, EI-MS	211
Salicylanilide	BuB, BenzB	GC	271
3'-Chloro-4'-(*p*-chlorophenoxy)-3-hydroxy-5-iodosalicylanilide	BuB, BenzB	EI-MS	271
3'-Chloro-4'-(*p*-chlorophenoxy)-3-hydroxy-5-iodosalicylanilide	BuB, BenzB	EI-MS	271
Salmefamol	BuB, BenzB	GC	58
Serine methyl ester	BuB	*I*, EI-MS	211
Tartaric acid	BuB	*I*, EI-MS	211
	BuB	*I*, EI-MS	224
Δ'-Tetrahydrocannabinolic acid	MeB, BuB, BenzB	*I*, EI-MS	270
o-Thymotic acid	BuB	*I*, EI-MS	224
	BuB	*I*, EI-MS	211

ephedrine to be achieved by GC[224,274]. The retention index values for some butaneboronate catecholamine derivatives are summarized in Table 26. The mass spectra of the catecholamine boronate derivatives have very favorable properties, characterized by strong molecular ions and a few prominent fragment ions indicating the substituents attached to the oxazaboralidine ring[224,274,275].

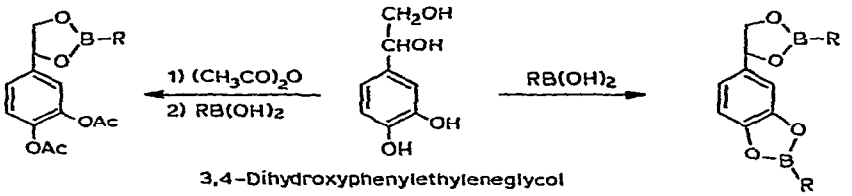
TABLE 26

RETENTION INDEX VALUES FOR SOME CATECHOLAMINE BUTANEBORONATE DERIVATIVES ON OV-17

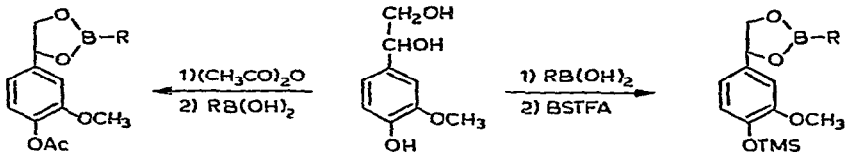
6 ft. glass column of 1% OV-17 on Gas-Chrom Q (100–120 mesh).

Catecholamine	*Column temperature (°C)*	*Retention index*
β-Hydroxyphenethylamine	140	1799
Norpseudoephedrine	140	1774
Phenylpropanolamine	140	1776
Pseudoephedrine	140	1782
Ephedrine	140	1796
Octopamine	170	2218
4-Deoxynoradrenaline	170	2203
Synephrine	170	2185
Phenylephrine	170	2171
Normetanephrine	190	2315
Metanephrine	190	2270
Norephinephrine	190	2478
Epinephrine	190	2438
3,4-Dihydroxynorephedrine	190	2450
Isoprenaline	190	2512

The catecholamine metabolite 3,4-dihydroxyphenylethyleneglycol (DHPG) was converted to its bis(boronate) derivative in twenty minutes at room temperature with methaneboronic or butaneboronic acid in 2,2-dimethoxypropane[276,277]. The bis-(boronate) derivative was stable in solution for at least twenty-four hours at room temperature. The phenolic groups can also be acylated in aqueous solution and after extraction the ethyleneglycol function converted to its methaneboronate or butaneboronate derivative by co-injection of the acylated DHPG extract and boronic acid into the GC[278]. The reaction in solution or by co-injection proceeds to the same extent but a calibration curve covering the range 3–30 μg was linear but did not pass through the origin. 3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) can be converted to its methaneboronate or butaneboronate derivative by reaction in 2,2-dimethoxypropane as solvent for 15 min at room temperature. Attempted silylation of the phenolic group with BSTFA resulted in a slow cleavage of the boronate group beginning after about one hour. The TMS derivative was prepared quantitatively without cleavage of the boronate group by sandwich injection of the boronate derivative and BSTFA into the gas chromatograph[278]. The phenol group can also be acylated in aqueous solution and after extraction into an organic solvent the boronate derivative of the ethyleneglycol group formed by co-injection of the extract and boronic acid into the gas chromatograph[279]. Formation of the acyl derivative, extraction and on-column boronation is recommended when a simultaneous analysis of DHPG and MHPG is required[276].



3,4-Dihydroxyphenylethyleneglycol



3-Methoxy-4-hydroxyphenylethyleneglycol

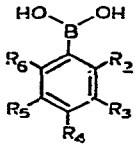
The catecholamines forming boronate derivatives suitable for their separation by GC are summarized in Table 27.

10.3. Boronic acids with electron-capturing properties

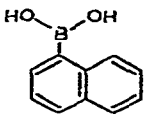
Boronic acids react specifically with bifunctional compounds to form derivatives stable to GC. The nature of the reaction provides some means of distinguishing this small group of compounds from the much larger pool of functionalized molecules in which they are found. The boronate reaction is specific as monofunctional compounds form derivatives which are not chromatographically stable. To take full advantage of this chemical specificity it is necessary to use a detector which is selective for the boronate derivatives and able to distinguish them from the general organic background. This combination of chemical specificity, chromatographic separating power and detector selectivity should provide a direct method of analyzing bifunctional compounds in complex mixtures (*e.g.*, physiological fluids) with the minimum of sample manipulation.

Hetero-element detectors have been developed for the selective detection of nitrogen, phosphorus and sulfur containing compounds. The scope of these detectors has been extended to embrace boron. The alkali flame ionization detector was adapted to the determination of sugar boronates[249] and steroid boronates[255] and the flame photometric detector to the determination of volatile boron hydrides[280]. Both detectors showed similar sensitivity with detection limits about 50-fold better than obtained with the universal flame ionization detector. This corresponds to a detection limit of about 10^{-8} g for the butaneboronate derivative of 2β,3β-dihydroxy-5α-cholestane[255]. The selectivity of the detector response towards boron compared to potential interfering organic compounds was not determined. With the rubidium bead NPD under conditions optimized for boron, only a poor selective response for boron could be obtained. For the benzeneboronate derivative of pinacol, the selective enhancement of the signal to boron over a hydrocarbon internal standard was about four-fold[281].

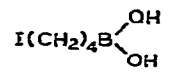
As an alternative approach to the specific detection of boron, boronic acids in which the organic substituent was modified to make it an electrophore have been prepared and used in conjunction with the selective and sensitive ECD[281–284]. The structures and appropriate abbreviations for these boronic acids are given below. Since their synthesis in the laboratory, 2,4-dichlorobenzeneboronic acid, 3,5-bis(trifluoro-



(I) → (VIII)



(IX)



(X)

(I)	$R_2 = R_3 = R_4 = R_5 = R_6 = F$;	pentafluorobenzeneboronic acid
(II)	$R_3 = R_5 = R_6 = H$, $R_2 = R_4 = Cl$;	2,4-dichlorobenzeneboronic acid (2,4-DCBB)
(III)	$R_3 = R_4 = R_5 = H$, $R_2 = R_6 = Cl$;	2,6-dichlorobenzeneboronic acid (2,6-DCBB)
(IV)	$R_2 = R_4 = R_6 = H$, $R_3 = R_5 = Cl$;	3,5-dichlorobenzeneboronic acid (3,5-DCBB)
(V)	$R_3 = R_5 = H$, $R_2 = R_4 = R_6 = Cl$;	2,4,6-trichlorobenzeneboronic acid (2,4,6-TCBB)
(VI)	$R_2 = R_3 = R_5 = R_6 = H$, $R_4 = Br$;	4-bromobenzeneboronic acid (4-BrBB)
(VII)	$R_2 = R_4 = R_5 = R_6 = H$, $R_3 = NO_2$;	3-nitrobenzeneboronic acid (3-NBB)
(VIII)	$R_2 = R_4 = R_6 = H$, $R_3 = R_5 = CF_3$;	3,5-Bis-(trifluoromethyl)benzeneboronic acid (3,5-BTFMBB)
(IX)	1-Naphthaleneboronic acid	(NAPB)
(X)	4-Iodobutaneboronic acid	(4-IBuB)

methyl)benzeneboronic acid and 4-iodobutaneboronic acid have become available commercially from Lancaster Synthesis (St. Leonardgate, Lancaster, Great Britain) and in the U.S.A. through the Alfa Products Division (Ventron Corporation, Danvers, Mass., U.S.A.).

The selection of suitable electrophores for attachment to boron presents several problems of a chemical and chromatographic nature. The choice of substituents is limited in the main to organic groups containing halogen atoms, nitro groups or conjugated systems to provide the necessary high response to the ECD[285,286]. As far as the halogens are concerned, the ECD has its highest response for iodine and declines through the series to fluorine, which is of course the reverse order of the volatility of their organic compounds on GC. To be of the widest possible use and particularly with high-molecular-weight compounds, it is of advantage if the boronic acid is capable of forming derivatives of high volatility. To provide good peak shape on GC and to reduce the possibility of column adsorption at low levels, the boronic acid should not contain polar groups in the organic substituent. Alkylboron compounds with halogen atoms on α, β or γ carbon atoms have poor hydrolytic and thermal stability. The thermally induced migration of halogens to boron with elimination of the organic group as an alkene takes place at temperatures likely to be employed for the separation of boronate derivatives by GC[198]. The boron–acyl bond is moisture sensitive and chemically very reactive[196]. The features discussed above influenced the choice of boronic acids (I) → (X) for evaluation as derivatizing reagents for GC with electron-capture detection.

All boronic acids except pentafluorobenzeneboronic acid can be used to derivatize a wide range of bifunctional compounds. Pentafluorobenzeneboronates were hydrolytically unstable and the presence of water (a product of the reaction to form the boronate derivative) resulted in elimination of pentafluorobenzene from the derivative[283]. Some bifunctional compounds did not produce derivatives with any boronic acid, these include bifunctional ketone compounds, thiol containing compounds, dicarboxylic acids and 1,3-substituted aromatic compounds. The boronate derivatives of aromatic carboxylic acids showed both solution and thermal degradation and are unsuitable for trace analysis. All derivatives were formed rapidly (within 15 min) at

TABLE 27

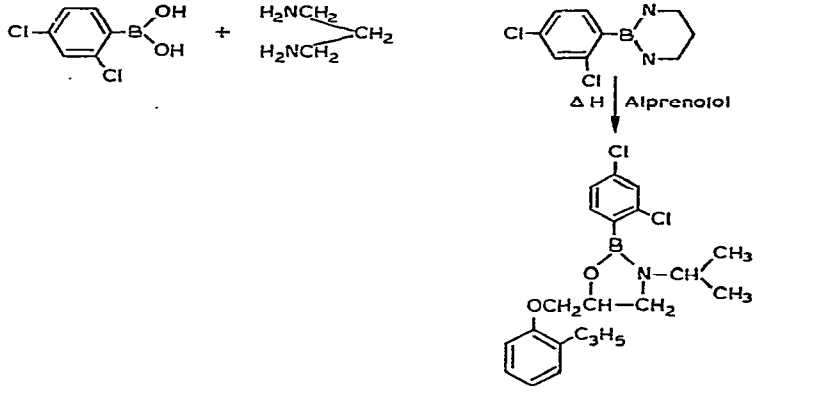
BORONATE DERIVATIVES USED IN THE GAS CHROMATOGRAPHY OF CATECHOLAMINES

Catecholamine	*Boronate derivative*	*Comments*	*Ref.*
4-Deoxynorepinephrine	BuB	*I*, EI-MS	274
3,4-Dihydroxynorephedrine	BuB	*I*, EI-MS	224
3,4-Dihydroxyphenylethyleneglycol	MeB, BuB	GC, EI-MS	276
	MeB, BuB	GC, EI-MS	277
Ephedrine	BuB, CHB, BenzB	GC, EI-MS	275
	BuB, CHB, BenzB	*I*, EI-MS	224
Epinephrine	BuB	*I*, EI-MS	274
	BuB	*I*, EI-MS	224
β-Hydroxy-β-phenylethylamine	BuB	*I*, EI-MS	224
	MeB, CHB, BenzB	*I*, EI-MS	274
	MeB, BuB, BenzB	GC, EI-MS	275
4-Hydroxyphenylethyleneglycol	MeB, BuB	GC, EI-MS	276
Isoprenaline	BuB	*I*, EI-MS	224
	BuB	*I*, EI-MS	274
Metanephrine	BuB	*I*, EI-MS	224
	BuB	*I*, EI-MS	274
3-Methoxy-4-hydroxyphenylethyleneglycol	MeB, BuB	GC, EI-MS	278
	MeB, BuB	GC, EI-MS	279
	MeB, BuB	GC, EI-MS	277
Methyl 2,3-dihydroxybenzoate	BuB	*I*, EI-MS	224
Methyl 3,4-dihydroxybenzoate	BuB	*I*, EI-MS	224
Neosynephrine	BuB	*I*, EI-MS	224
Norephedrine	BuB	*I*, EI-MS	224
	MeB, BuB, BenzB	GC, EI-MS	275
Norepinephrine	BuB	*I*, EI-MS	224
	BuB	*I*, EI-MS	274
Normetanephrine	BuB	*I*, EI-MS	224
			274
Norpseudoephedrine	BuB	*I*, EI-MS	274
	BuB	*I*, EI-MS	224
Octopamine	BuB	*I*, EI-MS	274
Phenylephrine	BuB	*I*, EI-MS	274
Pseudoephedrine	BuB	*I*, EI-MS	274
	BuB, CHB, BenzB	*I*, EI-MS	224
Synephrine	BuB	*I*, EI-MS	224
	BuB	*I*, EI-MS	274

room temperature in tetrahydrofuran as solvent by adding a molar quantity of the boronic acid to the bifunctional compound[281–284]. Alternative methods of preparing boronate derivatives were investigated using 2,4-dichlorobenzeneboronic acid and 4-iodobutaneboronic acid[287]. These methods include on-column reaction by sandwich injection, extractive derivatization by using a solution of the boronic acid in an organic solvent to extract and derivatize bifunctional compounds in aqueous solution and a cartridge reactor consisting of a modified injection port liner packed with 2,4-dichlorobenzeneboronic acid coated onto Chromosorb W into which test solutions were injected in the usual way. The sandwich injection technique was evaluated for the organic acids which showed signs of solution degradation. Poor reproducibility was obtained with salicylic acid, anthranilic acid and mandelic acid as with the solu-

tion technique. Many of the boronate derivatives were hydrolytically unstable and this limited the quantitative aspects of the extractive derivatization technique to those bifunctional compounds containing sterically crowded functional groups. Catechol and pinacol could be extractively derivatized in this way but other compounds such as salicyclic acid, 1,3-propanediamine, *o*-aminophenol and 3-amino-1-propanol either did not react at all or gave a very low yield of derivative. The cartridge reactor technique had the same range of application and limitations as the sandwich injection technique. Using the cartridge reactor a linear calibration curve over the range 0.1–3.0 μg was obtained for catechol with the FID.

A method has been developed for the determination of alprenolol by an on-column transborination reaction using the 2,4-dichlorobenzeneboronate derivative of 1,3-propanediamine as the transborination reagent[288]. Use of the transborination reagent had the advantage that excess reagent could be vented through the ECD without the large disruption in the baseline obtained by direct injection of 2,4-dichlorobenzeneboronic acid. Alprenolol extracted from plasma could be detected at the 0.1 ng level and a linear calibration graph was obtained over the range 0.5–70 ng.



2,4-Dichlorobenzeneboronate of Alprenolol

The reaction was rapid and selective as no peaks were obtained for a plasma blank and could be used routinely to monitor this drug in plasma. Initial studies provided little evidence for reaction between alprenolol and 2,4-dichlorobenzeneboronic acid in solution and the balance of probability favors the observation that this reaction either with the boronic acid or by transborination occurs principally in the injection port of the gas chromatograph.

All boronate derivatives investigated had good peak shape on GC .The 3-nitrobenzeneboronate derivatives exhibited poor peak shape on OV-17 but could be chromatographed as symmetrical peaks on OV-225 (ref. 282). Several boronate derivatives were decomposed on stainless steel columns and the use of nickel or glass columns was recommended for their separation[281–283]. Relative retention times for the chlorobenzeneboronate derivatives are summarized in Table 28, for the 3,5-bis-(trifluoromethyl)benzeneboronate and benzeneboronate derivatives in Table 29 and for the 4-iodobutaneboronate and 4-chlorobutaneboronate derivatives in Table 30. A comparison of the relative volatility of the boronate derivatives, Table 31 illustrates some interesting features. The 3,5-bis(trifluoromethyl)benzeneboronates are re-

TABLE 28

RELATIVE VOLATILITY OF CHLOROBENZENEBORONATES

90 × 0.2 cm I.D. nickel column of 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen flow-rate 60 ml min^{-1}. Compounds not forming boronate derivatives: 2,3-butanedione, 2,4-pentanedione, 2,5-hexanedione, 3-hydroxy-2-butanone, propane-1,3-dithiol; 2-anilinoethanol, 2-amino-2-hydroxymethyl-1,3-propanediol, ethylenediamine, methylguanidine; isophthalic acid, *trans*-1,2-cyclobutanedicarboxylic acid, oxalic acid, 2,3-pyridinecarboxylic acid, pyruvic acid, gallic acid, isoleucine, succinic acid, citric acid, malonic acid; pentane-1,5-diol, sorbitol; *m*-phenylenediamine, resorcinol, 3-aminophenol. Compounds forming 4-iodobutaneboronates but not 2,4-DCBB: 3-hydroxypropionic acid, 1,2-; 5,6-dianhydrogalactitol. Compounds forming 2,4-DCBB but not 4-iodobutaneboronates: 1,3-propanediamine.

Compound	*2,4-DCBB*	*3,5-DCBB*	*2,6-DCBB*	*2,4,6-TCBB*	*Column temperature (°C)*
Ethyleneglycol	0.40	0.32	0.32	0.51	140*
Pinacol	0.47	0.40	0.56	0.96	140
1,3-Propanediol	0.75	0.71	0.55	0.95	140
1,4-Butanediol	1.26	1.15	0.93	1.58	140
1,3-Cyclopentanediol	1.59	1.45	1.55	2.35	140
cis-1,2-Cyclohexanediol	2.54	2.13	2.41	3.93	140
Lactic acid	0.71	0.54	0.47	—	140
1,3-Propanediamine	0.90	1.30	—	—	140
3-Amino-1-propanol	0.79	1.30	0.78	—	140
2-Amino-1-butanol	0.70	1.00	0.86	—	140
Catechol	2.36	1.89	1.87	3.02	140
Phenyl-1,2-ethanediol	0.41	0.35	0.38	0.54	210**
o-Phenylenediamine	0.71	0.85	0.58	0.90	210
o-Aminophenol	0.32	0.43	0.33	0.52	210
D,L-Mandelic acid	0.63	0.53	0.53	0.68	210
Salicylic acid	0.64	0.64	0.50	0.67	210
Anthranilic acid	1.61	—	1.93	2.95	210

* Internal standard C_{20}, retention time = 5.7 min.
** Internal standard C_{28}, retention time = 5.6 min.

markably volatile having retention times significantly shorter than the benzeneboronates[281]. The 4-iodobutaneboronates have retention times approximately 1.8 times those of the benzeneboronates[284]. The 3-nitrobenzeneboronates and naphthaleneboronates have inconveniently long retention times for general use in GC[282,283].

The magnitude of the ECD response is remarkably temperature dependent and this parameter should be optimized for the comparison of detector response for different derivatives of a compound[289]. The minimum detectable quantity of pinacol as its boronate derivatives at their optimum detector temperatures are compared in Table 32[281]. With the exception of naphthaleneboronate all derivatives show a useful ECD response. The benzeneboronate derivative shows a moderate detector response which is enhanced by the introduction of halogen atoms. The position of the chlorine substituent effects the magnitude of the detector response and the 2,4-dichlorobenzeneboronate derivative was the most sensitive of the chlorobenzeneboronic acids evaluated. The 3,5-bis(trifluoromethyl)benzeneboronate derivative gave its maximum response at a low detector temperature and the detection limit was raised by a factor of 17 when the detector temperature was increased by 170°. High detector temperatures

TABLE 29

RETENTION TIME DATA FOR THE 3,5-BTFMB AND BenzB DERIVATIVES OF SOME REPRESENTATIVE BIFUNCTIONAL COMPOUNDS

Compound	*Derivative*		*Column temperature (°C)*
	BB	*3,5-BTFMBB*	
Ethylene glycol	1.00	0.33	80*
Pinacol	1.73	0.41	80
1,3-Propanediol	2.60	0.97	80
Lactic acid	2.39	—	80
1,4-Butanediol	1.25	0.47	100**
cis-1,2-Cyclohexanediol	2.84	0.79	100
1,3-Propanediamine	1.21	0.52	100
3-Amino-1-propanol	1.16	0.47	100
2-Amino-1-butanol	1.08	0.42	100
Catechol	2.45	0.74	100
Phenyl-1,2-ethanediol	0.67	0.19	160***
o-Phenylenediamine	1.43	0.57	160
o-Aminophenol	0.64	0.25	160

* Internal standard C_{14}, retention time = 3.0 min.
** Internal standard C_{16}, retention time = 3.8 min.
*** Internal standard C_{22}, retention time = 4.2 min.

are often preferred for the analysis of biological samples as this reduces detector contamination to the minimum.

The pinacol boronate derivatives are compared in terms of hydrolytic stability in Table 33. These data were gathered by partitioning an organic solution of the pinacol boronate against an aqueous sodium hydroxide solution for a fixed time interval

TABLE 30

RETENTION TIME DATA FOR 4-IBuB AND 4-ClBuB DERIVATIVES OF SOME REPRESENTATIVE BIFUNCTIONAL COMPOUNDS

Nickel column 90 × 0.2 cm I.D. packed with 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen flow-rate = 60 ml min^{-1}.

Compound	*4-Chlorobutane-boronate*	*4-Iodobutane-boronate*	*Column temperature (°C)*
1,2-Ethanediol	0.05	0.17	120*
Pinacol	0.09	0.26	120
1,3-Propanediol	0.10	0.31	120
1,3-Butanediol	0.21	0.60	120
Lactic acid	0.13	0.45	120
3-Aminopropan-1-ol	0.13	0.48	120
2-Aminobutan-1-ol	0.14	0.45	120
Catechol	0.36	1.00	150**
cis-Cyclohexane-1,2-diol	0.29	0.87	150
o-Phenylenediamine	0.59	1.47	200***
Mandelic acid	0.52	1.28	200
Salicylic acid	0.52	1.30	200

* Internal standard C_{19}, retention time = 9.6 min.
** Internal standard C_{20}, retention time = 3.7 min.
*** Internal standard C_{24}, retention time = 1.9 min.

TABLE 31

RELATIVE VOLATILITY OF THE BORONATE DERIVATIVES

Boronic Ester	*Relative volatility*
3,5-Bis(trifluoromethyl)benzeneboronates	0.3 ± 0.05
Benzeneboronates	1.0
4-Iodobutaneboronates	1.8 ± 0.5
4-Bromobenzeneboronates	3.9 ± 0.8
2,6-Dichlorobenzeneboronates	4.3 ± 2.0
2,4-Dichlorobenzeneboronates	4.7 ± 1.7
3,5-Dichlorobenzeneboronates	5.0 ± 1.1
2,4,6-Trichlorobenzeneboronates	6.9 ± 1.8
3-Nitrobenzeneboronates	11.7 ± 3.4
Naphthaleneboronates	18.5 ± 4.6

TABLE 32

RELATIVE ELECTRON-CAPTURE DETECTOR SENSITIVITY OF THE PINACOL BORONATES[281]

Detector A: Coaxial displaced cylinder type with a ^{63}Ni (8 mCi) source, operated in the pulse-modulated constant-current mode. Detector B: Coaxial cylinder type with a ^{63}Ni (30 mCi) source operated in the pulse mode with a pulse width of 4 μsec and a pulse period of 200 μsec.

Pinacol boronate	*Optimum detector temperature (°C)*	*Detector type*	*MDQ ($\times 10^{-12}$ g)*
2,4-Dichlorobenzeneboronate	325	B	2.0
	380	A	4.0
4-Bromobenzeneboronate	350	B	3.0
3,5-Bis(trifluoromethyl)benzeneboronate	180	A	3.0
2,4,6-Trichlorobenzeneboronate	380	A	4.0
3-Nitrobenzeneboronate	300	B	4.0
3,5-Dichlorobenzeneboronate	380	A	11.0
	325	B	9.0
4-Iodobutaneboronate	325	B	16.0
2,6-Dichlorobenzeneboronate	380	A	18.0
Benzeneboronate	200	B*	150.0
Naphthaleneboronate	350	B	2550.0

* This detector temperature is not optimum.

TABLE 33

RELATIVE HYDROLYTIC STABILITY OF PINACOL BORONATES

Contact time for hydrolysis experiment = 8.0 min; see ref. 281 for experimental details.

Pinacol boronate	*Percent hydrolysis*	
	1 N NaOH	*3 N NaOH*
Benzeneboronate	5	30
4-Iodobutaneboronate	5	22
4-Bromobenzeneboronate	15	31
2,6-Dichlorobenzeneboronate	24	—
3,5-Bis(trifluoromethyl)benzeneboronate	25	35
3,5-Dichlorobenzeneboronate	26	65
2,4,6-Trichlorobenzeneboronate	72	92
2,4-Dichlorobenzeneboronate	76	94

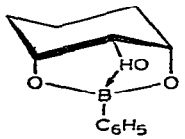
and then determining the percent of derivative which had been hydrolyzed. As can be seen the boronic acids show a wide range of hydrolytic stability varying from relatively stable to easily hydrolyzable.

An overview of the properties of the electron-capturing boronic acids lead to the recommendation that the four boronic acids, 2,4-dichlorobenzeneboronic acid, 4-bromobenzeneboronic acid, 4-iodobutaneboronic acid and 3,5-bis(trifluoromethyl)-benzeneboronic acid embodied a diverse range of advantages and some disadvantages which makes them the reagents of choice from the boronic acids tested for initial experimentation[281].

10.4 Miscellaneous chromatographic applications of boronic acids

Carbohydrates and acetylated carbohydrate derivatives were separated by paper electrophoresis in sulfonated benzeneboronic acid buffers at neutral pH[290]. The acetylated carbohydrates could not be separated in conventional buffer systems due to deacetylation or acyl migration occurring in alkali buffers and in acid buffers the derivatives had too low a mobility for adequate separation. Sugars with pyranosidic *cis*-1,2-diols and *cis*-1,3-diols showed little increase in mobilities compared to the furanosidic *cis*-1,2-diols which showed high mobilities. The spread of mobilities for the monosaccharides in the sulfonated benzeneboronic acid buffer enabled good separations to be obtained. Increased mobilities for some monosaccharides were also found for *ortho*- and *meta*-nitrobenzeneboronic acid complexes but the poor water solubility of these reagents rendered them unsuitable.

The addition of benzeneboronic acid to paper chromatographic solvents specifically enhances the mobilities of compounds possessing diol systems which form stable benzeneboronate derivatives[291–294]. With monosaccharides the most stable derivatives (*i.e.* those with the highest mobility) were formed between benzeneboronic acid and monosaccharides with *cis*-1,3 axial hydroxyl groups stabilized towards hydrolysis by the intervening equatorial hydroxyl group. The alditols (except glycerol) all form



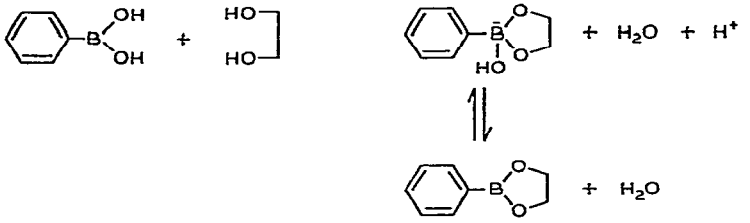
strong boronate complexes which enables the reduced form of the sugar to be easily separated from its parent. The configuration of 2-C-methyl-L-arabinose and L-ribose was assigned in part based on their ability to form complexes of enhanced mobility with benzeneboronic acid[294]. The paper chromatographic mobilities of cardenolides and bufadienolides with *cis*-1,3 -and *cis*-1,2-diols were increased in the presence of benzeneboronic acid and at higher concentration by dibenzeneboronic acid as well. Diols containing a tertiary hydroxyl group formed particularly stable complexes[293].

Phenanthreneboronic acid has been evaluated as a selective fluorescent reagent for the analysis of bifunctional compounds by HPLC and HPTLC[295]. Only compounds with sterically hindered hydroxyl groups yielded derivatives that were stable to reversed-phase HPLC or silica gel HPTLC. Hydrolysis of the derivatives occurred in most of the examples studied (*e.g.*, 1,2-ethanediol, 3-amino-1-propanol, *o*-phenylenediamine, catechol, salicylic acid etc.). The phenanthreneboronate derivative of pinacol was stable to HPTLC and could be detected by its fluorescence at 385 nm

when excited at 313 nm down to the 0.6-ng level. The fluorescence intensity decreased with time in the form of a shallow curve possibly due to either a slow chemical reaction on the plate or due to oxygen quenching. The insect moulting hormones, ecdysone and ecdysterone, were separated as their phenanthreneboronate derivatives by HPTLC and qualitatively identified by fluorescence measurement. The method was sufficiently selective for the detection of ecdysones in crude insect samples.

10.5. Immobilized boronic acid phases for liquid column chromatography

Ion-exchange chromatography of sugars in the form of their anionic boronate complexes is a well established technique for their separation and analysis. The aromatic boronic acids are also capable of complexing with sugars in a manner similar to boric acid[296,297]. The presence of the aromatic group in the boronic acid makes for a simplification in the complexation reaction as only 1:1 complexes can be formed.

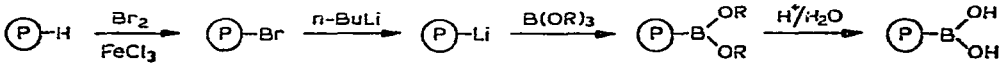


Experimental evidence for complex formation is derived from the increase in acidity observed when a solution of benzeneboronic acid is added to certain diols with the correct spatial arrangement for complex formation[298]. The formation of the anionic complex is favored in alkaline solution and the pH dependence of the equilibria between complex formation and dissociation enables a chromatographic separation to be devised. As benzeneboronic acids are Lewis acids, their acidity is affected by the electron density on the boron atom and the anionic boronate complex should be stabilized by electron-withdrawing groups in the aromatic ring. For example, the pH value for 100% formation of the benzeneboronate complex of D-glucose and D-fructose is close to pH 9.0 whereas for the corresponding 3-nitrobenzeneboronate complex it is close to pH 7.9 (ref. 299).

Immobilization of the boronic acid group by incorporation or attachment to a polymer network provides chromatographic materials which were used to isolate or separate bifunctional compounds. The sample is passed through the column material at a controlled pH favoring formation of the anionic complex which then remains attached to the polymer while other non-complexed compounds are washed away (some non-specific adsorption of compounds to the polymer network may also occur). The complexation reaction is reversible by adjustment of the pH in a stepwise fashion or with a continuous pH gradient for separation based on the stability of the anionic complex or by elution at acid pH, at which all anionic complexes are dissociated for isolation purposes. In practice, the binding capacity of the boronic acid resin or gel is highly influenced by the ionic strength of the solution and also by the amount of interfering non-bifunctional compounds. To date, most work has concerned the use of boronic acid resins or gels for the separation of sugars and nucleosides (via the ribose substituent) or catecholamines in aqueous media including biological fluids such as serum, amniotic fluid and urine.

Ion-exchange resins with boronic acid groups were first prepared by Solms and Deuel[300]. Acid-catalyzed condensation polymerization of *m*-aminobenzeneboronic acid, *m*-diaminobenzene and formaldehyde was used. The resulting material was treated with formic acid–hydrochloric acid or with pyridine and acetic anhydride to cap the free amine groups. An alternative resin was prepared by condensation of formaldehyde and *m*-aminobenzeneboronic acid with the weakly basic anion-exchange resin Duolite A114. Resins of the above type were generally chromatographically inefficient and contained residual ion-exchange sites but could be used to separate simple sugar mixtures.

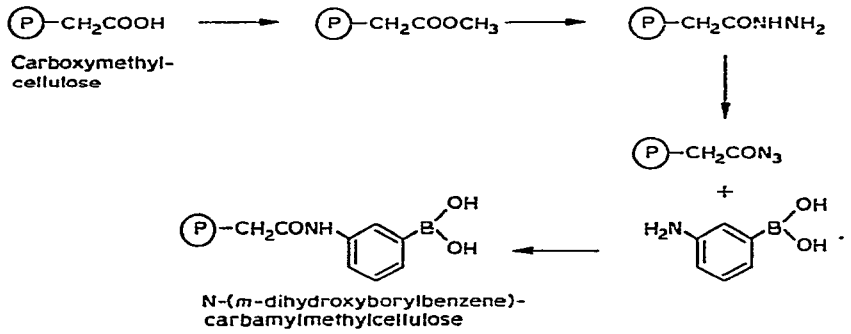
The successful synthesis of *p*-vinylbenzeneboronic acid led to the production of polymeric materials either by catalyzed self condensation or by co-polymerization with styrene or diallylmaleate[301–304]. These materials were obtained in the form of air stable powders or "popcorn" polymers but were not tested specifically as chromatographic materials. It was noted however that the polymeric boronic acid materials would bind 2-aminoethanol and *o*-diaminobenzene[302]. The iminodiethyl derivative of 4-vinylbenzeneboronic acid was polymerized with a mixture of divinylbenzene, ethylvinylbenzene and azobisisobutyronitrile (free radical catalyst) to form a lightly cross-linked gel which swelled in alkaline aqueous solution and thus allowed monosaccharides to enter the gel matrix[305]. The boronic acid gel was successfully used to separate simple mixtures of monosaccharides and the effect of pH and temperature on retention factors was determined. The use of the polymer to improve the yield of D-fructose from D-glucose (the former resulting from the action of alkali on the latter) was also demonstrated using a closed system in which the product from a conversion reactor was passed continuously through the boronic acid column to selectively remove fructose from the reaction mixture and the eluent enriched in glucose fed back to the reactor. 4-Vinylbenzeneboronic acid has also been polymerized[306] by (a) copolymerization with styrene; (b) copolymerization with N,N′-methylenebis(acrylamide); (c) copolymerization with acrylamide and N,N′-methylenebis(acrylamide) and (d) interstitial homopolymerization on porous polystyrene beads (Chromosorb 102). The boronic acid polymers were evaluated chromatographically for the separation of L-DOPA from L-tyrosine. Polymer (d) was found to be superior in performance to the other three polymers (a) → (c). At pH 8.0 the breakthrough capacity for a 21.0 × 1.0 cm column containing 5.36 g of polymer (d) was 120 ml (130 μmole g^{-1}) for L-DOPA compared to about 10 ml of solution for L-tyrosine. Elution of complexed L-DOPA and column regeneration was achieved by using 0.1 *M* acetic acid as the eluent. An investigation of a wide range of related hydroxyacids and phenols indicated that the column selectively complexed only *o*-dihydroxyaromatic compounds and that the binding of these compounds to the boronic acid resin was strongly pH dependent. Macroreticular porous polystyrene-divinylbenzene resins (*e.g.*, Amberlite XE-305) can be functionalized to contain the boronic acid group by the route shown below[307].



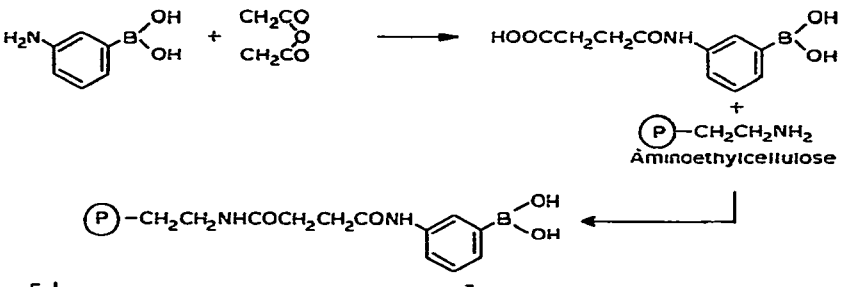
P = Polymer matrix

Polymers containing 1–2 mmole g^{-1} of boronic acid groups were prepared in this way and used in the solid phase synthesis of glycosides. The glycoside was coupled to the polymer in dry pyridine by azeotropic removal of water and was removed at the end of the synthesis by suspension in acetone–water (4:1). A similar polymer was used to separate *cis*-1,2-cyclohexanediol from its *trans* isomer by batchwise extraction. The *cis* isomer reacted selectively with the boronic acid resin and was then separated from the *trans* isomer which remained in solution by filtration[308].

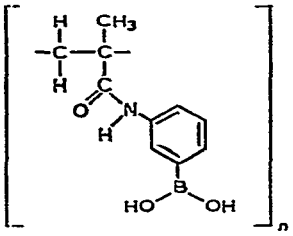
For the study of large biological molecules, the use of resins or gels with pores presenting restricted access to the boronic acid group could be a problem. For the analysis of polynucleotides, Weith *et al.*[309] have described the synthesis of boronic acid groups attached to cellulose powder. In their synthesis, carboxymethylcellulose was converted to the azide form and coupled with *m*-aminobenzeneboronic acid to form N-(*m*-dihydroxyborylbenzene)carbamylmethylcellulose containing about 0.2 mmole $-B(OH)_2$ g^{-1} of dry cellulose.



The material obtained in this way still contained a relatively high concentration of carboxylic acid groups (approximately 33% of the carboxymethyl groups were converted to the boronic acid) which was undesirable for some applications. In an alternative synthesis, *m*-aminobenzeneboronic acid was converted into N-(dihydroxyborylbenzene)succinamic acid and then condensed with aminoethylcellulose in the presence of N-cyclohexyl-N′-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate as the activating agent. The N-[N′-(*m*-dihydroxyborylbenzene)succinamyl] aminoethylcellulose material contained approximately 0.6 mmole of $-B(OH)_2$ g^{-1} of cellulose corresponding to substitution of 60% of the amino groups present in the starting material[310]. At neutral pH the remaining amine groups are not protonated and

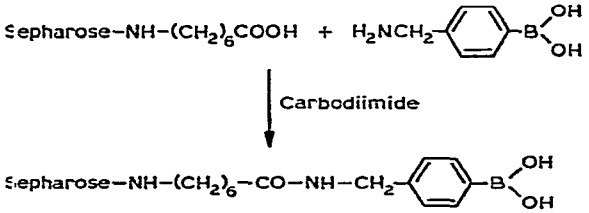


do not interfere in the formation of the boronate complexes. Columns of the above type when used to separate nucleosides showed a strong dependence of the elution order on the presence of a *cis*-glycol system in the sugar moiety, the pH of the eluting solvent, the ionic strength of the eluting solvent and the nature of the nucleoside base. Sugars which are bound most strongly are those with the highest proportion of *cis*-glycol groups especially if the glycol exist in a coplanar conformation. The retention of polynucleotides was found to be dependent on similar factors[309–311].

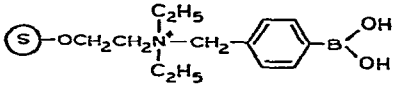


Schott has described the synthesis of a dihydroxyborylsubstituted methacrylic acid polymer with the general structure shown below[312]. Depending on the reaction conditions, the boron content of the polymers varied from 0.11 to 0.58%. These polymers have been shown to be suitable for the separation of ribonucleosides from deoxyribonucleosides (not retained), ribonucleotides carrying 2'- or 3'-*cis*-diol groups from the corresponding deoxynucleoside 5'-phosphate and ribonucleoside 2'- or 3'-monophosphates, the separation of oligonucleotide mixtures and the separation of aminoacylated tRNA from unchanged tRNA[313]. The capacity of the boronate gels seems to depend on the nature and particularly the chain length of the oligonucleotides. This difference probably arises from the reduced accessibility of the boronic acid groups in the interior parts of the gel matrix to molecules of large diameter. An improved method for the synthesis of polyacrylamide-boronate gel, based on the approach described by Weith *et al.*[309], has been proposed by Hagen and Kuehn[314]. Aminoethyl Bio-Gel P-150 was converted to the N-succinylaminoethyl derivative and coupled to *m*-aminobenzeneboronic acid at pH 4.7 in the presence of L-ethyl-3(3-dimethylaminopropyl)carbodiimide. Under these conditions coupling was almost complete and gels containing between 0.9–1.1 mmole of $-B(OH)_2$ g^{-1} were obtained. This boronic acid gel was used to separate ATP (retained through the *cis*-diol moiety on the ribose ring) from cyclic AMP which does not bind to the boronate gel.

p-Methylaminebenzeneboronic acid has been attached to CH-Sepharose (a Sepharose derivative containing ε-aminocaproic acid residues with free carboxyl groups) activated by N-cyclohexyl-N'-[2-(4-morpholinyl)ethyl]carbodiimide *p*-toluenesulfonate[315]. Gels prepared in this way contained 5–10 μmole $-B(OH)_2$ ml^{-1} of swollen Sepharose and were used to purify Subtilisn BPN', an extracellular serine

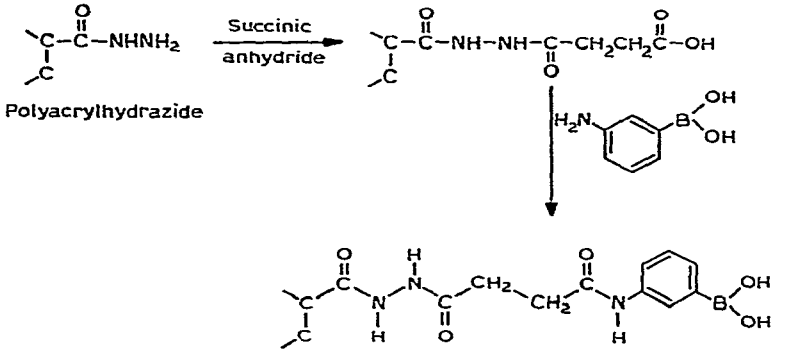


proteinase of "*Bacillus subtilis*". Binding was assumed to be through the functional groups of the amino acid constituents of the enzyme. A column of the boronate gel was used to provide a 42-fold purification of a culture medium extract. A 1.9-fold purification of a commercial source of the enzyme was obtained on the same column. Sephadex A-25 was converted to its N,N-diethyl-N-(*p*-methylbenzeneboryl)amino-ethyl derivative and was evaluated for complexing with *cis*-diol compounds[316,317]. The polymer was highly specific for the binding of polyols, carbohydrates, nucleosides and nucleotides over a wide pH range. The chromatographic behavior of carbohydrates was controlled by their structure and conformation which was also responsible for the different stabilities of the boronate anionic complexes generated[317,318].



The monosaccharides D-ribose, D-mannose, D-arabinose were separated by a 0.01 *M* $[(C_2H_5)_3NH]HCO_3$ buffer at pH 8.4, lactose, D-glucose and D-ribose with a 0.01 *M* sodium acetate buffer pH 6.5 and D-glucitol and D-fructose with the previous buffer at pH 5.0 (ref. 318). The same boronic acid gel was also used for the separation of nucleosides and mononucleotides[317], oligonucleotides[319] and in the isolation of pure aminoacylated nucleotides[319].

Polyacrylhydrazide can be succinilated and then condensed with *m*-aminobenzeneboronic acid in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide to give a polyacrylamide boronic acid gel which has been extensively evaluated for the analysis of nucleosides[320]. The synthesis of the gel has been optimized by subsequent workers and described in detail[321]. Compared to the synthesis by Uziel *et al.*[320] a finer mesh polyacrylylhydrazide with a lower concentration of hydrazide groups (1.2 mmole g^{-1} dry weight) was used as starting material to give a polymer gel with decreased



shrinkage when either the pH or the ionic strength of the eluent was changed. A synthetic mixture of nine nucleosides at the 10 nmole level each, had recoveries of 88–101% when put on a polyacrylamide boronic acid gel column in an ammonium acetate buffer of pH 8.8 and eluted with 0.1 *M* formic acid. The columns could be regenerated and used many times by washing with formic acid and re-equilibrated before use with ammonium acetate buffer pH 8.8. The capacity limit of 0.8 ml of polyacrylamide boronic acid gel was established as 40–50 μmoles of nucleosides at

pH 8.5 to 9.4 (ref. 322). It seems likely that complex formation between the diol of the nucleosides and the boronic acid group is not the only process controlling the binding of the nucleosides. The order of elution from the column places nucelosides with an intrinsic negative charge (exclusive of boronic acid–diol complexation) at the front of the elution pattern and those nucleosides that are cations or potential cations at the rear[320]. For the common nucleosides found in biological fluids, pseudouridine is the least tightly bound and its retention is strongly influenced by the pH of the solution used to load the sample on the column and by the concentration of other substances present in urine besides the ribonucleosides[320,322,323]. A complete analytical scheme comprising preliminary isolation of the nucleosides from biological fluids using a polyacrylamide boronic acid gel column with elution and collection of the nucleoside fraction and subsequent separation and analysis by HPLC has been described and used for the analysis of nucleosides in urine, blood, amniotic fluid and tRNA hydrolysates[321,322,324]. A similar boronic acid gel was used as a preliminary isolation procedure for 5-fluorouridine in urine[325] and 5-fluoro-2'-deoxyuridine in plasma[326] which were determined by capillary column GC after derivatization. The polyacrylamide boronic acid gel has also been used to analyze cysteinyldopas, dopamine and catecholamines which were separated into three separate fractions using a stepwise pH gradient[327]. Adsorption of all catecholic compounds on the immobilized benzeneboronate gel takes place at neutral or alkali pH (*i.e.* 8.0 or higher) and at slightly lower pH the extent of adsorption becomes dependent on the compound concerned. The gel was specific for the catecholamine structure as neither tyrosine nor 3-methoxytyramine were adsorbed even at high pH.

Polymers bearing structural analogies to biological receptors were prepared containing a boronic acid group locked into a fixed position in a polymer cavity[328]. Vinylboronic acid was condensed with a template molecule, *p*-nitrobenzene-α-D-mannopyranoside and then polymerized with a co-monomer (also a cross-linking agent) in the presence of an inert solvent to obtain a macroporous polymer[329]. High cross-linking ensured a rigid polymer with fixed cavities that contain the boronic acid group (after selective removal of the template) locked in a fixed stereochemical arrangement. The polymer was used in HPLC to analyze the optical isomers of the template with very high specificity (separation factors $\alpha = 1.05$–2.32). Unfortunately, the columns themselves were inefficient as measured by their plate height and also showed excessive tailing which tended to offset the advantages gained by the high separation factors. The high specificity of the polymers was demonstrated by the lack of resolution of racemates other than the template.

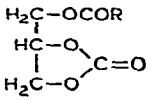
11. MISCELLANEOUS REAGENTS FOR THE DERIVATIZATION OF BIFUNCTIONAL COMPOUNDS

Melatonin reacts with pentafluoropropionic anhydride under mild conditions to form a 3,3-spirocyclic indole derivative[330,331]. The ethyl and propyl amide analogues

Melatonin

of melatonin were also cyclized but the isobutyryl amide gave a low yield of the cyclic derivative and the pivaloyl amide underwent displacement of the pivaloyl group with replacement by a pentafluoropropionyl group. The structures of all derivatives were confirmed by physical means[330]. The melatonin derivative could be detected in plasma at the 1 pg ml^{-1} level by negative chemical-ionization MS[331,332].

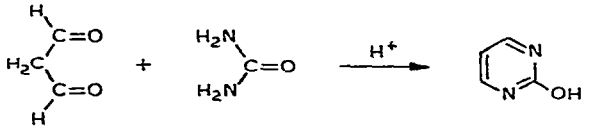
The mass spectral properties of a miscellaneous series of cyclic sulfite and carbonate derivatives have been described[333]. The cyclic carbonate derivatives were formed by reaction with phosgene in yields of 50–70%, some of which were purified by GC. Cyclic carbonate derivatives have been used for the separation by GC and identification by MS of the 1-monacyl-glycerol-2,3-carbonates[334,335]. The derivatives were prepared by the cyclization of the glycerol 1-β,β,β-trichloroethylcarbonate in pyridine at 80° (ref. 334). In an alternative synthesis, 1-O-benzylglycerol was converted to its 2,3-carbonate derivative by treatment with potassium hydrogen carbonate and diethylcarbonate.



1-monoacylglycerol-2,3-carbonate

The mass spectra of the 1-monoacylglycerol-2,3-carbonates were characterized by the presence of a molecular ion and daughter fragment ions of high abundance identifying the acyl group and the presence of the cyclic carbonate group[335].

Malonaldehyde was converted to 2-hydroxypyrimidine in approximately 90% yield by reaction with excess urea in acid solution[336]. The hydroxypyrimidine was isolated by ion-exchange chromatography and converted to its TMS ether derivative for GC with identification by MS.

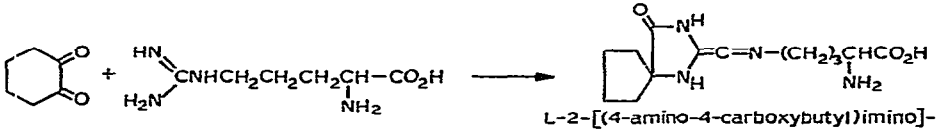


Malonaldehyde Urea 2-hydroxypyrimidine

Primary aliphatic and aromatic amines react to form the N-substituted 2,5-dimethylpyrrole derivatives quantitatively with 2,5-hexanedione by heating the amine with a five-fold excess of 2,5-hexanedione in ether at 60° for 30 min in a vessel protected from light with nitrogen bubbling through the solution[337]. Aromatic amines react much slower than aliphatic ones and sterically hindered amines like 2,6-dimethylanaline require heating for 2 h for complete reaction and *tert.*-butylamine does not react quantitatively under any conditions. Diamines such as *p*-phenylenediamine are

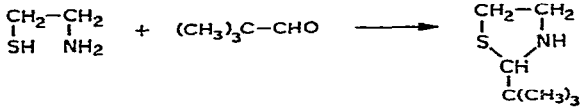
N-substituted 2,5-dimethylpyrroles

rapidly converted to the monoamine derivative and only slowly to the di derivative. The presence of water in the reaction mixture lowers the yield of derivatives. Under the influence of light, oxygen or acid the reaction mixture deteriorates producing discoloration and a low yield of derivative. Arginine reacts with 1,2-cyclohexanedione in aqueous base to form a cyclic imidazolidinone derivative which was used for the detection of arginine residues in peptide digests by paper chromatography and electrophoresis[338]. A similar derivative was formed with benzil.

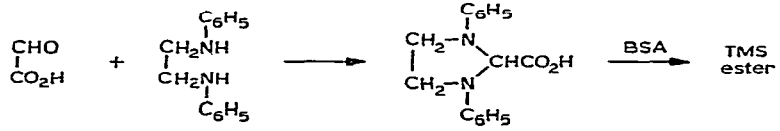


L-2-[(4-amino-4-carboxybutyl)imino]-5,5-cyclotetramethylene-4-imidazolidinone

Aminothiols react rapidly at neutral pH and at room temperature with pivalaldehyde to form cyclic thiozolidine derivatives which are stable to GC[339]. The reaction was complete within 10 min when a small amount of basic ion exchanger in the bicarbonate form was added as a catalyst. Thiolamino acids and disulfides extracted from biological fluids could be separated by GC after methylation and condensation with pivalaldehyde.



Glyoxalic acid was converted to an imidazolidine-2-carboxylic acid derivative by treatment with N,N'-diphenylenediamine in pyridine at room temperature for 2 h which could be gas chromatographed as its TMS ester[340].



Glyoxalic acid

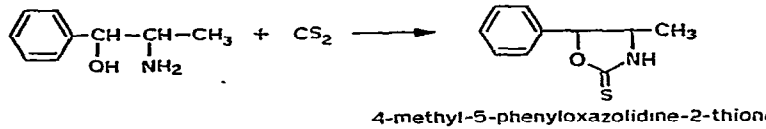
1,3-diphenylimidazolidine-2-carboxylic acid

Diclofenac was converted to its indole derivative by heating at 70° for 75 min in a 0.5% (v/v) solution of sulfuric acid in 2,2,2-trifluoroethanol[341]. The derivative was stable to GC and could be determined with an ECD at the nanogram level.

Diclofenac

Phenylpropanolamine reacts with carbon disulfide at room temperature in a non-quantitative reaction to form 4-methyl-5-phenyloxazolidine-2-thione[342]. The

derivative could be gas chromatographed with or without formation of its N-TMS derivative.



4-methyl-5-phenyloxazolidine-2-thione

12. ABBREVIATIONS

AFID	Alkali flame ionization detector
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BenzB	Benzeneboronate
BrBB	Bromobenzeneboronate
BSA	N,O-Bis(trimethylsilyl)acetamide
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
BTFMBB	Bis(trifluoromethyl)benzeneboronate
BuB	Butaneboronate
t-BuB	*tert.*-Butaneboronate
CHB	Cyclohexaneboronate
CI	Chemical ionization
ClBuB	Chlorobutaneboronate
CMDMCS	Chloromethyldimethylchlorosilane
CMTMDS	1,3-Bis(chloromethyl)-1,1,3,3-tetramethyldisilazane
DCBB	Dichlorobenzeneboronate
DHPG	3,4-Dihydroxyphenylethyleneglycol
DMCS	Dimethyldichlorosilane
DMDAS	Dimethyldiacetoxysilane
DMMCS	Dimethylmethoxychlorosilane
DMSO	Dimethyl sulfoxide
DOPA	Dihydroxyphenylalanine
ECD	Electron-capture detector
EI	Electron impact
EPTD	Ethylphosphonothioic dichloride
FID	Flame ionization detector
FPD	Flame photometric detector
FR	Flow-rate
GC	Gas chromatography
HFAA	Hexafluoroacetylacetone
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
HMDS	Hexamethyldisilazane
I	Retention index according to Kováts
IBuB	Iodobutaneboronate
IR	Infrared spectroscopy
IS	Internal standard
MDQ	Minimum detectable quantity

MeB	Methaneboronate
MHPG	3-Methoxy-4-hydroxyphenylethyleneglycol
MS	Mass spectrometry
MTH	Methylthiohydantoin derivatives
MU	Methylene unit value
NAPB	1-Naphthaleneboronate
NBB	Nitrobenzeneboronate
NMR	Nuclear magnetic resonance spectrometry
NPD	Rubidium bead nitrogen–phosphorus detector
OR	Optical rotation
PAPTH	*p*-Phenylazophenylthiohydantoin derivatives
PFPTH	Pentafluorophenylthiohydantoin derivatives
PTH	Phenylthiohydantoin derivatives
tRNA	Transfer ribonucleic acid
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TMDS	1,1,3,3-Tetramethyldisilazane
TMS	Trimethylsilyl ether/ester
UV	Ultraviolet spectroscopy

13. ACKNOWLEDGEMENTS

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14. SUMMARY

Only a few reagents capable of forming cyclic derivatives with bifunctional compounds have been described in the chromatographic literature. Some reagents are selective for a particular compound, whereas others such as the boronic acids are more generally applicable to a wide range of bifunctional compounds. All reagents provide a high degree of selectivity through the chemical discriminatory power of the reaction employed and some reagents have been developed which have high detector discriminatory power as well. These are of particular interest for the analysis of a few components (bifunctional compounds) in a complex matrix without the need for a tedious amount of sample clean-up. When it is necessary to isolate bifunctional compounds from complex sources, columns containing immobilized boronic acid groups have been used and advantage taken of the reversibility of the boronate complexation reaction for attachment and later removal of the desired bifunctional compound.

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