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COMMENTARY

MICRO-ANALYTIC DETECTION AND STRUCTURE ELUCIDATION OF ESTER-GLYCOSIDES

KAREL P. M. HEIRWEGH* and FRANS COMPERNOLLE†

*Laboratory of Hepatology, Department of Medical Research, University of Leuven, B-3000 Leuven, Belgium and †Department of Macromolecular and Organic Chemistry, University of Leuven, B-3030 Heverlee, Belgium

Many endogenous and xenobiotic substances, or their metabolites, are transformed into monosaccharidic derivatives by conjugation with one or more sugar moieties before their excretion into bile or urine [1–5]. In many instances ester-, ether- or *N*-glycosides are formed. Although in mammals the conjugating moiety is most often D-glucuronic acid [3] *in vivo* conjugation with other sugars such as D-glucose [4–13], D-ribose [14–16], D-xylose [9, 10] or *N*-acetyl-D-glucosamine [6] may be important, depending on species and tissue.

Methods for structure elucidation of monosaccharidic glycosides, in particular glucuronides, have been known for a long time. A detailed and critical account of the chemistry of glucuronic acid and of glucuronides, up to 1966, has been given by Marsh [17]. More recently, Bakke [18] discussed procedures for extraction, column chromatography and mass-spectral identification of glucuronides.

It often happens that in the course of a biochemical (e.g. an enzyme assay) or a biological study (e.g. conversion *in vivo* of a drug) only small amounts of pure glycoside become available. Provided that the metabolic pathways involved are already known to some extent, chemical characterization of the reaction products obtained may be considered primarily a problem of confirming or disproving a number of postulated structures. It is the purpose of the present commentary to show that, in particular for ester-conjugates, much structural information and sometimes even confirmation of postulated structures can be achieved by applying simple chemical treatments and thin-layer chromatographic separations.

General approach

In attempts to determine the structures of glycosides it is practical to distinguish the structure of (a) aglycon and (b) sugar moiety, and (c) the nature of the glycosidic linkage.

(a) Provided that a suitable method for cleavage of the conjugating bond is available, confirmation of aglycon structure is less difficult than it may look. Indeed, in general, the number of choices is limited. For instance, when a drug or other compound is injected into an animal with subsequent excretion of a derived glycoside the aglycon will either be the injected substance itself or, if changed, it is most likely to be a closely related derivative [3]. Considering what is known about the metabolic fate of the compound tested, or of chemically related compounds, possible reaction products may be known or can be guessed at, and suitable reference materials may thus be available. Also, in the course of an enzymic synthesis of a glycoside *in vitro*, one is not entitled to assume that the acceptor substrate and the sugar portion (usually derived from a sugar nucleotide) must of necessity appear unchanged in the final product, but here the number of choices will probably be more limited than in the former case. Identification of aglycon on a micro-scale by t.l.c. thus will rely crucially on availability of suitable reference compounds and of adequate separation techniques.

(b) Few types of sugars have been found in 'detoxication' glycosides. For mammals, known conjugating residues are D-glucuronic acid, D-glucose, *N*-acetyl-D-glucosamine, D-ribose and D-xylose, but others may be detected.

(c) Ease of cleavage of the conjugating bond by chemical means depends on the type of linkage. In slightly alkaline medium (pH 11–11.5) at room temperature ester–glycosides are hydrolysed completely after 5–30 min [17, 19–22]. At neutral pH, treatment with hydroxylamine yields typical aglycon hydroxamates [21, 22]. Cleavage reactions transforming the aglycon part of ester-conjugates into carboxylic acid derivatives such as hydroxamates, amides or esters can be exploited to determine the locus of attachment of sugar. Ether–glycosides and, in particular, those containing an aromatic aglycon are, in general, quite stable to hydrolysis by dilute acid or alkali. Complete cleavage under more drastic conditions often leads to considerable destruction of aglycon and sugar [17]. Exceptionally, hydrolysis by dilute acid [17, 23] or alkali [17] has been observed. Some *N*-glycosides may be cleaved at neutral to slightly acid pH [17, 19].

Many glycosides can be hydrolysed under gentle conditions by treatment with specific hydrolases. Detailed discussions of enzyme specificity and of specific inhibition of hydrolysis by aldonolactones have been given [25, 25]. In general, pure glycosidases show absolute specificity with respect to anomer configuration at C-1. Glycosidases can hydrolyse a wide range of glycosides with different aglycons, in particular *O*-glycosides [24]. In a recent study [26] one out of four *S*-glucuronides tested resisted hydrolysis by β-glucuronidase and two *S*-glucosides were not attacked by β-

glucosidase; *N*-glycosides may also resist enzymic hydrolysis [12, 19]. Relatively high substrate specificity is observed with regard to the sugar moiety. For instance, β-glucuronidase from *Escherichia coli* only hydrolyses β-glucuronides and β-galacturonides, compounds with a furanose ring structure being hydrolysed more slowly than the pyranuronosides [24]. The range of the carbohydrate substrates depends on the source of enzyme. For example, almond emulsin hydrolyses both β-galactosides and β-glucosides, whereas the latter glycosides are not attacked by β-galactosidase from barley [25].

Hydrolysis of glycosides is inhibited by structurally related aldonolactones [24, 25]. For instance, glucaro-(1–4)-lactone inhibits β-glucuronidase but has no action on other glycosidases (including α-glucuronidase); β-glucosidase is inhibited by glucono-(1–5)-lactone. By taking advantage of inhibition by aldonolactones, hydrolysis of glycosides by glycosidases offers powerful tools (i) to establish the anomer configuration in a sensitive way and (ii) to obtain information about the sugar residue.

Determination or confirmation of structures of glycosides require simple laboratory equipment and can be applied to very small amounts of glycosides e.g. the amount that can just be detected as a single spot on a thin-layer plate, or slightly more. Chemical reactions considered are simple and require commonly available reagents. The procedures are of four types:

(i) *Preparation and t.l.c. of derivatives of intact glycoside.* Non-destructive detection is required at stages where intermediate derivatives are to be isolated for further reaction.

(ii) *Determination of the conjugating linkage and sugar residue by treatment of glycosides with specific hydrolases.* Tests must be done with and without specific inhibitor. With labile conjugates spontaneous hydrolysis should also be assessed.

(iii) *Alcoholysis in slightly alkaline medium.* This reaction allows easy confirmation of aglycon structure and determination of the locus of attachment of sugar residues in ester-glycosides.

(iv) *Cleavage on thin-layer plates of ester-conjugates by alkaline vapour.* The same information as mentioned in (iii) above, but also information about the sugar residue, can be obtained. After application of the glycoside to the thin-layer plate any components or derivatives never leave the plate so that potential losses are minimized. Non-destructive detection procedures are needed when aglycon and carbohydrates are developed separately with appropriate consecutive solvent systems.

Some procedures outlined below have been worked out with ester-glycosides of bilirubin-IXα and, in more detail, of azodipyrrole derivatives of bilirubin-IXα. Conditions of cleavage are likely to apply to a wide range of ester-conjugates; application to a few types of other conjugates is shown.

Equipment and reference compounds

Only standard laboratory equipment such as chromatographic tanks, glass-stoppered centrifuge tubes, an ordinary laboratory centrifuge (max. speed about 3000 rev./min), is needed. We have made extensive use of silica gel plates containing a fluorescence indicator (DC-Kieselgel F254. 5715/0025; Merck A.G., Darmstadt, Germany). By illumination of the plates with a source of ultraviolet light, spots of colourless, aromatic compounds appear as dark areas against a coloured background of transmitted light. Chromatographic references such as methyl esters of compounds suspected to be the aglycon are easily prepared by treatment with ethereal diazomethane; amides can be obtained by further treatment of the methyl esters with ammonia. Many glycosides can be synthesized *in vitro* by incubating enzyme (usually a microsomal preparation from liver) with suspected aglycon material and a suitable UDP-sugar [1, 2, 4–6, 27]; only the β-D-glycosides are obtained. Chemical synthesis of glycosides has been reviewed by Marsh [17].

Analytic procedures

For t.l.c. of derivatized and underivatized glycosides, and of substances suspected to be aglycons, and their amides and methyl esters, the present authors have used extensively neutral solvent mixtures composed of benzene/ethyl acetate (place tanke under well-ventilated hood), chloroform/methanol and chloroform/methanol/water [28]. By changing the proportions of the components a wide range of polarities can be covered. Some typical solvent mixtures mentioned in Table 1 cause minimal migration of pentoses on silica gel plates, and none of neutral and acid hexoses [28]. With ester–glycosides alkaline solvents should not be employed. The textbook on thin-layer chromatography by Stahl [29] is a most useful source of information with regard to solvent systems, detection procedures, etc. With silica gel plates containing a fluorescence indicator a spot of 3 nmoles of glycoside containing *o*-nitrophenol, *p*-nitrophenol or phenolphthalein as aglycon could easily be localized. With a spray reagent based on naphtharesorcinol [29] sugar released from 3–10 nmols of azodipyrrole glucuronide could already be detected, detection of neutral sugars being somewhat more sensitive.

T.l.c. of derivatives of glycosides. Glycoside is submitted to t.l.c. with known material(s) as reference(s). After elution and treatment with diazomethane t.l.c. is again performed with untreated and suitably treated references. In general, the presence of one or more carboxyl groups will be evident by increased hydrophobicity of the methyl esters. With an excess of diazomethane, formation of methyl esters of dissolved carboxylic acid is nearly instantaneous [28, 30]. Spots of treated material are scraped from the plates and treated for 0.5–4 hr at room temperature with 0.5 ml of pyridine/acetic acid (2:1, v/v) for formation of acetates [28, 31] and the fully acetylated derivatives are again submitted to t.l.c. alongside with appropriate reference compounds. Free amino and hydroxyl groups will react under these conditions.

Since solvent systems of quite different polarities will be needed to obtain migration of free glycoside, its methyl ester (if applicable) and of fully acetylated compound, identical chromatographic behaviour at the various stages of analysis at least proves close structural similarity. Information about the type of linkage (alkali-labile or not?) and, if it is labile, about the nature of the aglycon, can be obtained by submitting eluted acetylated glycoside to alcoholysis in alkaline medium.

Elution of glycoside, in particular hexuronide, from silica gel may be difficult and incomplete. This is the

case with azodipyrrole glucuronide. Methanol and pentan-2-one were unsatisfactory. Good results were obtained by eluting the spots, on silica gel powder, with a small volume of pentanone/formamide (4:1, v/v). The formamide was removed by washing with glycine/HCl buffer, pH 2.7 (0.4 M-HCl) and the water-saturated pentanone extract was treated with ethereal diazomethane [30].

For formation of the methyl ester the solvent has critical importance [30]. With ester–glycosides methanol produces erratic results; sometimes near-complete methanolysis with formation of aglycon methyl ester occurred. Chloroform and dry pentanone yielded poor conversions whereas pentanone or tetrahydrofuran containing some water were satisfactory. Complete solution of test compound can easily be checked by centrifugation. The sequence, methyl ester formation followed by acetylation is probably better than the reverse, as it avoids possible side reactions such as lactonization. Multiple carboxyl groups can be detected by treating glycoside with a limiting amount of diazomethane, thus yielding several methyl ester spots on t.l.c. On re-elution and further treatment with reagent they should yield the same fully methylated derivative.

Obviously, completely unequivocal identification cannot be achieved in this way. However, in many cases this should be possible with near-certainty provided several derivatives can be chromatographed sequentially against appropriate reference compounds. Indeed, separation of sugars is often improved by substitution of hydrophilic groups [29]. Data obtained with *p*-nitrophenyl glycosides suggest that the same principle applies. In particular, the anomeric isomers of glycosides were differentiated quite easily after acetylation (Table 1). Isomeric glycosides with identical anomeric configuration could also be separated. Other separations on silica gel have been obtained for the glucoside and galactoside of estrogen [32], the glucuronide, glucoside and xyloside of azodipyrrole [28] and a variety of bilirubin-IXα glycosides [31, 33, 34]. The four *O*-acyl positional isomers of azodipyrrole glucuronide are easily separated as their methyl esters [30].

Hydrolysis with glycosidases. After enzymic incubation of pure glycoside the extent of hydrolysis is assessed by extraction and t.l.c. of aglycon and remaining glycoside. As glycosidases are often contaminated by related hydrolases, parallel incubations with and without specific enzyme inhibitors are mandatory. For glycosides with labile conjugating linkage the extent of spontaneous hydrolysis should also be assessed to allow more accurate determination of the effects of enzyme and inhibitor.

Incomplete hydrolysis does not necessarily reflect heterogeneity of the glycoside or inadequate enzyme activity. (i) For practical work complete solution of glycosides with highly hydrophobic aglycons must be checked carefully e.g. by centrifuging the incubation mixtures before addition of enzyme. Unsolubilized material is likely to be hydrolysed very slowly, if at all, and could thus reappear unchanged after extraction of the incubation mixture and t.l.c. In studies with azodipyrrole β-D-glucuronide and β-D-glucoside the compounds were first dissolved in dimethyl sulphoxide (final concn in the incubation mixtures 2.5–8 per cent, v/v). No untoward effects on the activities of β-glucuronidase (from bovine liver) and on β-glucosidase (almond emulsin) were noted. (ii) With ester–glycosides lack of hydrolysis can also be due to the presence in the preparations of non-C-1 isomers [35]. These may arise by sequential migration of the aglycon acyl group from position 1 of the sugar to positions 2, 3 and 4 during storage at neutral to slightly alkaline pH and during stasis in the body [30, 35, 36]. Heterogeneity of clofibric acid glucuronides isolated from urine and serum, and resistance to enzymic hydrolysis of some isomers [37] has been explained in a similar way (E. M. Faed, personal communication). (iii) As already mentioned, *S*- and *N*-glycosides may less easily be hydrolysed than *O*-glycosides.

In trying to obtain information about the sugar residue one should consider the limits of established specificity for the enzyme preparation used [24, 25]. In general, hydrolysis of a glycoside by a hydrolase and inhibition of the reaction by a specific inhibitor will

Table 1. R_f values of glycosides of *o*- and *p*-nitrophenol

Compound	A	B
o-Nitrophenol β-D-glucopyranoside	0.51	0.31
o-Nitrophenol β-D-galactopyranoside	0.49	0.34
p-Nitrophenol α-L-fucopyranoside	0.66	0.67
p-Nitrophenol β-L-fucopyranoside	0.64	0.55
p-Nitrophenol α-D-xylopyranoside	0.63	0.61
p-Nitrophenol β-D-xylopyranoside	0.62	0.51
p-Nitrophenol β-D-mannoside	0.55	0.46
p-Nitrophenol α-D-glucopyranoside	0.50	0.49
p-Nitrophenol β-D-glucopyranoside	0.50	0.37
p-Nitrophenol β-D-galactopyranoside	0.46	0.39
p-Nitrophenol-*N*-acetyl-β-D-glucosaminide	0.46	0.024
p-Nitrophenol β-D-glucuronide	0.093	0.46
p-Nitrophenol β-D-galacturonide	0.070	0.42

T.l.c. was on silica gel plates. Free glycosides were developed with (A) chloroform/methanol/water (65:25:3, by vol.) (over 18 cm), and treated glycosides with (B) benzene/ethyl acetate (3:1, v/v) (over 14.5 cm). Treatment consisted of acetylation preceded by methyl ester formation for the hexuronides. The amounts chromatographed corresponded to 10 μg of free glycosides.

limit the choice to a few types of sugar conjugates. In practice, some possible structures may be considered unlikely. For instance, β-galacturonides (β-glucuronidase from *E. coli*) and β-galactoside (almond emulsin) are substrates of the enzymes mentioned but have not been found in bile or urine. Similarly, glycosides with a furanose ring structure have not yet been detected.

Methanolysis of ester–glycosides. Conversion at 0° and 25° of 20 μM-azodipyrrole glucuronide into azodipyrrole methyl ester at various concentrations of NaOH is illustrated in Fig. 1. At 0° methanolysis of 10–50 μM-glucuronide in the presence of NaOH, 5 mg/ml, required 4–5 min reaction; at 25° complete conversion was already attained after 1 min. Even after 10 min at 25°, 20 μM-glucuronide in pure methanol, and 47 μM-azodipyrrole in methanol containing NaOH, 5 mg/ml, were completely intact. Cleavage of ester–glycosides can also be achieved with other alcohols such as ethanol or propan-1-ol by prolonging the reaction time. No cleavage was observed when 20 μM solutions of ether–glycosides (β-D-glucoside of *o*-nitrophenol; α- and β-D-glucoside and *N*-acetyl-β-D-glucosaminide of *p*-nitrophenol; β-D-glucuronide of phenolphthalein), in methanol containing NaOH, 5 mg/ml, were kept at room temperature for 1–10 min before analysis.

The following procedure which is in routine use has given satisfactory results with the glucuronide, glucoside and xyloside of azodipyrrole [28]. Glycoside dissolved in 0.5 ml of methanol is mixed at room temperature with 0.5 ml of methanol containing NaOH, 10 mg/ml. After 30–60 sec an excess (2–8 ml) of glycine/HCl buffer, pH 2.7 (0.4 M-HCl) is added. The products are extracted with 0.5–2 ml of chloroform or pentan-2-one and submitted to t.l.c. with any likely aglycons and their methyl esters as references.

Formation of an ester of the aglycon shows the initial ester-linkage. Identification of the aglycon is strengthened if additional functional groups are available for derivative formation, e.g. hydroxyl groups. Conditions for conversion established with azodipyrrole glycosides are probably very similar for a wide range of ester–glycosides. The pH adopted for extraction may have to be adapted to individual needs. If the aglycon contains carboxylic acid ester groups multiple derivatives can arise by transesterification if the ester groups differ from the alcohol used for dissolving the glycoside.

Establishment of ester-linkage and determination of sugar and aglycon after application of a glycoside to a thin-layer plate. Garay *et al.* [38] treated azodipyrrole glucuronide on chromatographic paper with ammonia vapour to release the sugar for subsequent identification. We have adapted this technique for work with thin-layer plates.

Cleavage of ester bonds. Cleavage can be performed at room temperature with the alkaline vapours produced by (a) concentrated ammonia (27 per cent, w/v [28], (b) water-free methanol saturated with gaseous ammonia [35], or (c) the mixture, water-free methanol/trimethylamine (3:1, v/v) (our unpublished work) (Table 2). A chromatographic tank is provided at the bottom with a layer of fluid a, b or c. Introduction of some pieces of glass rod allows subsequent positioning of the thin-layer plates without direct contact with the fluid. For further safety a small layer (about 1 cm) of chromatographic supporting material may be scraped from the plate near the edge which will be in contact with the glass rods. Spots of pure glycoside and reference compounds (sugars, aglycons and derivatives to be tested) are applied to the plate. This is then introduced into the tank and left overnight at room temperature. Considerable cleavage of ester–glycosides already occurred after 2 hr treatment (Table 2). As expected, the ether–glycosides mentioned above resisted alkaline treatment.

T.l.c. of non-sugar reaction products. After removal in an air stream of excess volatile reagents from the plate, additional references may be applied. The presence and sequential development of various aglycon derivatives (methyl ester, amide and free aglycon) will strengthen identification as identical chromatographic behaviour can be observed for 2 or 3 substances (Table 2). The composition of derivatives obtained varies typically with the nature of the alkaline vapour used for cleavage.

Non-destructive detection of aglycon products

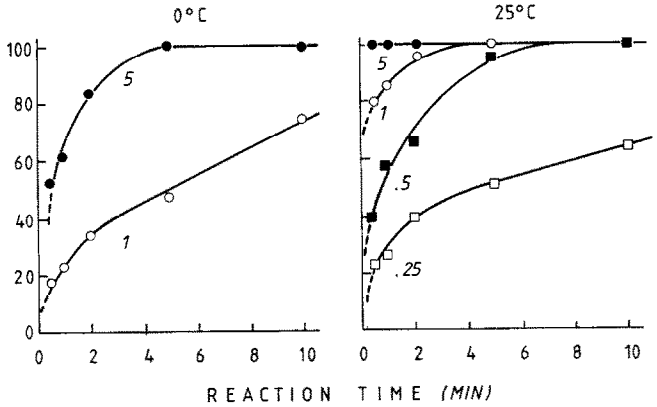

Fig. 1. Alkaline methanolysis of an ester–glucuronide. Azodipyrrole glucuronide (40 μM) dissolved in methanol was treated with an equal volume of methanol containing NaOH at 0° or 25° for the indicated periods of time. Methanolysis was stopped by adding an excess of glycine/HCl buffer, pH 2.7 (0.4 M-HCl) and the products were extracted with pentan-2-one and submitted to t.l.c. [28]. Results are expressed as percentage of initial glucuronide converted into azodipyrrole methyl ester. The numbers in the figure indicate the final concentrations of NaOH (mg/ml).

Table 2. Cleavage by alkaline vapour of the ester-linkage of azodipyrrole glucuronide on silica gel

	Compounds detected after treatment (percentage of total azo colour)															
	CH_3OH/NH_3				H_2O/NH_3						$CH_3OH/N(CH_3)_3$					
Vapour phase	6.3		29		6.3			29			6.3			29		
Amount of glycoside (nmoles)																
Reaction time (hr)	2	17	2	17	2	6	17	2	6	17	2	6	17	2	6	17
Derivative of azodipyrrole																
Methyl ester	61	77	43	71							32	47	64	32	44	61
Amide	10	9	16	11	68	72	78	63	64	70						
Free aglycon	2	2	3	3	22	21	17	24	26	21	1	0.4	9	2	1	12
Breakdown products					2	2	1	1	2	1						
Remaining glucuronide	28	12	35	15	8	4	4	13	8	8	67	52	27	66	54	26

should be possible if the same plate is to be used for subsequent detection of released sugars. Pentoses moved slightly in chloroform/methanol/water (65:25:3, by vol.) but not with the other, less polar, solvent systems in Table 1.

T.l.c. of released sugars. The authors have used extensively mixtures of propan-1-ol/water [29] for t.l.c. of neutral and acid sugars. Typical compositions in work with commercially available, untreated (17:3, v/v) or silane-treated silica gel plates (9:2, v/v) are indicated in parentheses. Apparently, treatment with ammonia vapour (room temperature, overnight) did not affect a variety of sugars (including D-glucuronic acid, D-galacturonic acid, D-glucose and D-xylose) as their R_F values were unchanged and no additional sugar-positive spots appeared. D-glucuronic acid and D-galacturonic acid are well separated on silane-treated plates [35].

General comments

The major merit of the techniques suggested is their simplicity with respect to equipment needed and manipulations involved. Non-destructive localization of aromatic aglycons and glycosides on silica gel plates containing a fluorescence indicator and chemical detection of released sugars are of adequate sensitivity. Alkaline alcoholysis and cleavage of glycosides on thin-layer plates by treatment with alkaline vapour primarily are simple diagnostic tests for ester-conjugates. When suitable reference compounds are available (amide or ester of aglycon, depending on the treatment applied) these can be used to establish the locus of attachment of sugar to the aglycon. Identification of a variety of sugars by t.l.c. after cleavage of the glycosidic bond by alkaline vapour is simple and straightforward.

O-ester and *O*-ether glycosides and some other types of sugar conjugates are hydrolysed by glycosidases that are specific for the anomer configuration at C-1. When enzymic hydrolysis of glycosidic conjugates is applied with due regard to possible causes of over-reaction (heterogeneity of some enzyme preparations) or lack of reaction (incomplete solution in incubation mixtures; presence of acyl-shifted ester-conjugates) this technique offers a valid and sensitive way to establish the anomer configuration at C-1 and to limit the number of possible sugar structures. To reach valid conclusions it is mandatory to test enzymic hydrolysis of glycoside in presence and absence of an appropriate, specific enzyme inhibitor.

It may be rewarding, in the future, to pay closer attention to possible conjugation of drugs and endogenous compounds with sugars other than glucuronic acid. Several instances have been mentioned above. In most studies of the excretion of administered aglycons, only a few animal species have been investigated. Given the proper combination of species and acceptor substance unusual routes of conjugation may be found. For instance, xyloside has been detected recently in bile of 5 out of 11 species, as yet only for a single aglycon, bilirubin-IXα [10]. Alternatively, some non-glucuronide conjugates may primarily serve as metabolic intermediates [13] or may have other roles, but may not be excreted in bile or urine.

The techniques of sequential derivative formation suggested are primarily of a confirmatory nature. When an aglycon is unknown its structure must be determined by some well-established organic–analytical route such as mass spectrometry. Even when metabolic transformation of an administered drug is well explored a considerable number of intermediates may be formed before conjugation and excretion [3]. Chromatographic analyses should therefore be interpreted with caution. However, provided appropriate derivatives of starting material and of possible intermediates and glycosides are available, different chromatographic behaviour of unknown and references may enable one to rule out a number of structures. Alternatively, identical chromatographic behaviour of unknown and a reference, at various stages of derivatization, in suitably discriminating solvent systems will confirm identical or at least closely related structures.

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