Pergamon 0031-9422(95)00314-2 Phytochemistry, Vol. 40, No. 2, pp. 433–437, 1995
Elsevier Science Ltd
Printed in Great Britain
0031-9422/95 $9.50 + 0.00

PROTOCATECHUALDEHYDE AND OTHER PHENOLS AS CELL WALL COMPONENTS OF GRAPEVINE LEAVES

BERNHARD WEBER, LIENHARD HOESCH and DORA M. RAST*

Department of Plant Biology, University of Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland

(*Received in revised form* 28 *November* 1994)

Key Word Index—*Vitis vinifera*; Vitaceae; grapevine; protocatechualdehyde; protocatechuic acid; hydroxycinnamic acids; quercetin-β-glucuronide; cell wall; leaf development; phytopathological significance.

Abstract—Upon washing with dilute bicarbonate solution, quercetin glucuronide as well as the monotartaryl esters of 4-coumaric, caffeic and ferulic acids were released from extensively solvent-purified cell wall material (CWM) of *Vitis vinifera* leaves. Drastic alkaline hydrolysis additionally yielded protocatechualdehyde and protocatechuic acid. Digestion of bicarbonate-washed CWM with a commercial esterase liberated protocatechuic and 4-coumaric acids, but no protocatechualdehyde. The latter was, however, solubilized upon incubation of the same CWM with pectinase ('Macerase') and subsequent alkaline hydrolysis, together with some of the two phenolic acids. In contrast to this, the analogous set-up with cellulase was almost ineffectual, inasmuch as only traces of these acids were released. Thus, quercetin glucuronide and the tartaric esters of the hydroxycinnamic acids are only loosely associated with the cell wall, whereas part of the 4-coumaric and protocatechuic acids as well as the protocatechualdehyde are covalently ester-bonded. The phenolic acids probably act as the acyl parts of esters with polysaccharidic hydroxyl groups, and the aldehyde as the hydroxyl part of esters with glucuronic or galacturonic acid moieties. The contents of extractable protocatechualdehyde and protocatechuic acid decreased by about 60% from the early to the late exponential phase of leaf growth. The possibility is discussed that the decrease in covalently wall-bound protocatechuic compounds during leaf development is causally related to the age-dependent establishment of resistance to fungal disease.

INTRODUCTION

Some hydroxycinnamic acids, particularly ferulic and 4-coumaric acids, have long been known to be regular, although seemingly not ubiquitous, components of the cell wall in all major groups of vascular plants ([1, 2]; for further references, see [3–6]). In contrast to this, the occurrence of wall-associated low molecular phenols of other types, e.g. flavonoids [4] and hydroxybenzoic acids [5], appears to be rather erratic. The cell walls from graminaceous species can, additionally, harbour phenolic aldehydes [7].

Any of these wall phenols may be covalently bound to polysaccharidic material. Such binding must evidently reduce the susceptibility of the wall fabric to hydrolytic cleavage, particularly if conjugation is followed by phenolic coupling reactions (cf. [8]). Wall-bound phenols are, therefore, likely to play a major role not only in the regulation of extension growth [8, 9], but also in the development of the plant's resistance against lysis by microbial pathogens. The present study was aimed at identifying cell-wall phenols other than lignin precursors in *Vitis vinifera*. This has been reported to lack wall-associated ferulic, 4-coumaric and 4-hydroxybenzoic acids altogether [2] (for ferulic acid, see also ref. [10]). It was thought, therefore, that grape material could contain other, perhaps more specific, surface-located phenols. A preliminary account of this work has been presented earlier [11].

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Identity of wall-associated phenols

The presence of the phenols listed in Table 1 was indicated following drastic alkaline treatment of extensively purified cell wall material (CWM; Experimental and Table 2), the separation of components of the fraction obtained by TLC and HPLC (UV photodiode detection), specific colour tests for phenols (DANA) and aldehydes (DNPH), and comparison with test compounds. Further analysis was by UV and ^{1}H NMR spectroscopy (see Experimental).

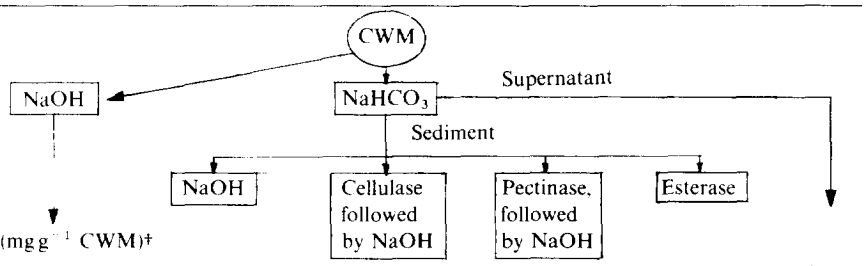
The quercetin glucuronide (Que-glcA) as well as 4-coumaric, caffeic and ferulic acids have been described previously as constituents of *V. vinifera* [12–14], not, however, as wall-associated phenols. *In situ*, the hydroxycinnamic acids are probably present as tartaryl

Table 1. Characteristics of phenols isolated from cell wall material of grapevine leaves

Compound*	TLC† R_f Solvent A	TLC† R_f Solvent B	Colour reaction DANA‡	Colour reaction DNPH‡	HPLC R_t (min)	UV absorption λ_{max} (nm)
Pro-COOH	0.30	0.42	+	–	3.60	222; 258; 294
Pro-CHO	0.28	0.46	+	+	5.30	231; 280; 311
Cou-COOH	0.56	0.58	+	–	12.30	232; 311
Caf-COOH	0.33	0.41	+	–	8.90	228; 280*sh*; 325
Fer-COOH	0.75	0.51	+	–	14.45	233; 278*sh*; 323
Que-glcA	0.00	0.02	+	–	17.60	255; 305*sh*; 355

*For abbreviations, see Table 2.
†Solvent A, CH_2Cl_2–HOAc (4:1); solvent B, toluene–diisopropyl ether–formic acid (5:4:1).
‡DANA, diazotized 4-nitroaniline; DNPH, 2,4-dinitrophenylhydrazine.

Table 2. Isolation and quantitation of phenols present in CWM of grapevine leaves



Compound*	(mg g^{-1} CWM)†					
Pro-COOH	0.35	+	tr	+	+	tr
Pro-CHO	0.30	+	–	+	–	–
Cou-COOH	0.03	+	tr	+	+	++
Caf-COOH	0.07	–	–	–	–	++
Fer-COOH	0.01	–		+	–	++
Que-glcA	0.15	–	–	–	–	+

*Abbreviations: Pro-COOH, protocatechuic acid; Pro-CHO, protocatechualdehyde; Cou-COOH, 4-coumaric; Caf-COOH, caffeic; Fer-COOH, ferulic; Que-glcA, quercetin 3-glucuronide.
†Results are given for one of five independent experiments with leaves of IS3 (cf. caption to Fig. 1).
‡Detected as tartaryl esters.
tr = Trace.

esters [12–14], since they were easily extractable with dilute alkali ($NaHCO_3$). 4-Coumaric acid was, however, also present in a form tightly bound to the CWM, from which it was liberated only upon drastic alkaline hydrolysis (Table 2) or by enzymic treatment (see below). Roughly 60% of total NaOH-hydrolysable phenols was accounted for by protocatechuic acid and protocatechualdehyde (Table 2), which both occur exclusively in the wall compartment of the leaf cells [11]. Whereas association of protocatechuic acid with the wall compartment has been described earlier (from *Ginkgo biloba* [5]), the existence of the aldehyde as an integral and major cell wall constituent represents a phytochemical novelty. The occurrence, *per se*, of protocatechualdehyde has been reported previously from grape seeds (after acid hydrolysis [15]) as well as from *Cichorium intybus* [16] and *Solanum tuberosum* [17], but without a consideration of its cellular compartmentation. Accounting for the high susceptibility to oxidation of the diphenol and the special care taken here to prevent this (see Experimental), protocatechualdehyde may not be such a rare plant cell wall component as it appears at present (cf. [18]).

Type of sub-compartmentation and linkage partners of wall-associated phenols

Whether quercetin glucuronide and the hydroxycinnamic acid tartaryl esters (Table 2) are held in the wall by cavity sequestration, simple hydrogen bonding with polysaccharides or complexation with protein [19, 20] cannot, of course, be decided on the basis of the present data. To determine the functionalities by which the tightly wall-associated phenols are bonded, the action was studied of esterase, pectinase (Macerase) and cellulase on CWM pre-washed with $NaHCO_3$ (Table 2). Upon digestion with esterase, 4-coumaric and protocatechuic acids were liberated. Treatment with a pectinase preparation (displaying also some hemicellulase activity), followed by alkaline hydrolysis of the residue, additionally afforded protocatechualdehyde as well as ferulic acid. The appar-

ent release of the latter is probably an artefact, since ferulic acid was found to be a substantial contaminant (*ca* 50 ng per sample analysed) in the enzyme preparation used to solubilize the phenols (for details, see Experimental). The same set-up as followed in the pectinase experiments, but substituting the latter by cellulase, was almost ineffectual (Table 2). With their carboxyl group(s) the two acids probably participate in ester linkages to hydroxyl groups of matrix components, as holds for 4-coumaric and ferulic acids in spinach leaves [9]; hemicelluloses and pectins are the likely partners [8, 21]. The different response of wall-bound aldehyde to treatment with the esterase used, which is a carboxylic ester hydrolase (EC 3.1.1.1) acting only on esters of low molecular weight carboxylic acids [22], requires comment. Whereas the acids can participate in phenol ester linkages by both the acyl and the hydroxyl groups, the aldehyde must, of necessity, be conjugated with the acyl functionality of a wall constituent. If this is a polymer containing, e.g. galacturonic or glucuronic acid residues, digestion with the esterase would be ineffectual, as has indeed been observed (Table 2). As the low-molecular acyl parts of an ester functionality, the phenolic acids, however, can be released by treatment with this esterase.

Developmentally related changes in contents of wall-bound phenols and their potential phytopathological significance

When leaves of the grape cultivar studied here have reached some 80% of their final surface area, as represented by material of insertion site six (IS6, with IS9 leaves fully grown), they become practically resistant to infection by *Botrytis cinerea* [11]. Such age-related resistance of grapevine leaves also holds against *Uncinula necator* [23] and *Plasmopara viticola* [24]. Covalently wall-bound low-molecular weight phenols may contribute to the plant's preformed resistance against fungal pathogens by both their intrinsic fungitoxicity [25, 26], which presumably becomes effective only upon their release under the influence of an invader, and the chemical changes brought about in the wall fabric upon their cross-linking with primary cell wall constituents [8, 21]. It appeared, therefore, of interest to check whether there is some correlation between the development of resistance and the pool sizes of the major wall-bound phenols (Table 2). Considering that a truly accurate quantitation of catechols cannot be achieved, due to their easy oxidation, particularly at high pH values (> 10), as evidenced visibly by the rapid, irreversible browning of the hydrolysate upon exposure to the air, it was, therefore, considered more meaningful to present the maximal values obtained (Table 2, Fig. 1) rather than means with large standard deviations.

As shown in Fig. 1, there was a clear-cut decrease in the concentration of alkali-hydrolysable protocatechuic acid and aldehyde with increasing age (and resistance) of the leaves, which levelled off towards the end of growth. The analogous situation held for 4-coumaric acid, the concentration of which was zero in CWM from IS6 leaves [11]. Three phenomena may be considered to be

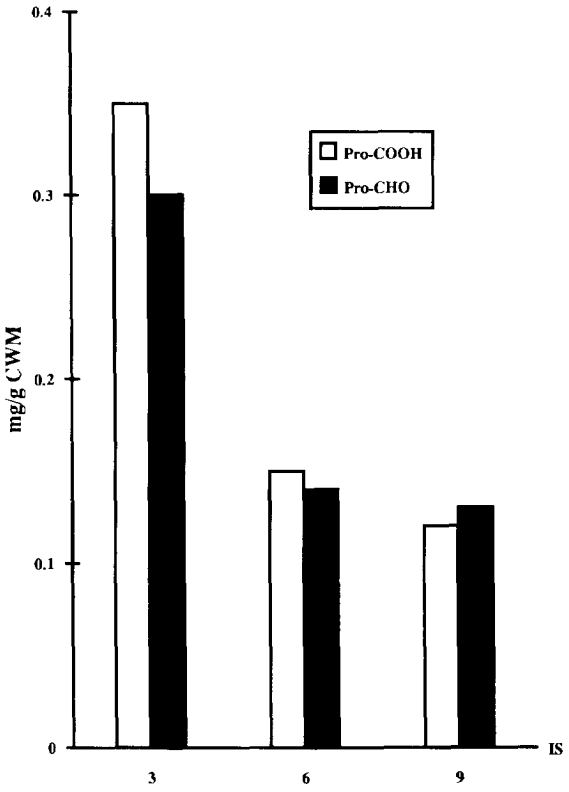


Fig. 1. Developmentally related changes in the concentration of the major cell wall phenols of *V. vinifera* leaves, i.e. protocatechuic acid (Pro-COOH) and protocatechualdehyde (Pro-CHO). IS refers to the insertion site of the leaf on the plant, IS1 corresponding to the youngest, just unfolded leaf, and IS9 to a fully developed one.

responsible for the reduction observed in these pool sizes: (1) degradation *sensu* ring fission (cf. [27]), (2) translocation into the protoplastic compartment, and (3) transformation into a non-hydrolysable polymer. Since the ability to catabolize phenols appears to be rare among green plants [27, 28], and since none of the catecholic compounds was detectable in the soluble fraction of the cells at any stage of leaf development [11], (3) remains the likely case. It is, therefore, suggested that the decreases observed in the pool sizes of the three phenolics releasable from their covalent association with other wall components by alkali treatment (Fig. 1) reflect the partial conversion of these into an inherently non-hydrolysable material. Thus, the following events may well represent successive steps towards the establishment of the developmentally acquired preformed defence system of grapevine leaves against microbial disease: (i) the initial build-up of a pool of covalently wall-associated phenolics which act as cross-linkers between major wall polymers, (ii) their gradual release from their conjugation by ester hydrolases, (iii) the oxidation of the phenols thus set free by catecholase/phenolase (EC 1.10.3.1/1.14.18.1), and, thence, (iv) the copolymerization of phenolic radicals thus generated as well as of suitable non-phenolic co-

reactants to insoluble material, which, by the very nature of its structural features, is intrinsically recalcitrant to degradation by hydrolases. It is implicit in this scheme, and also fully compatible with the particulars of 'hypersensitive lignification' occurring upon fungal attack [29], that protocatechuic acid and protocatechualdehyde remaining after the developmentally regulated deposition of insoluble polymer can be released upon microbial attack and serve as the substrates of 'hypersensitive melanization'. The hypothesis presented here is but an extension of the one proposed earlier [26, 30] for plants storing mainly covalently bound phenyl propanoid lignin precursors in the wall.

EXPERIMENTAL

Plant material. Healthy leaves of *V. vinifera* L. (cv. Riesling × Silvaner) were collected from freeland-grown vines and stored at −20°.

CWM. Cell walls were isolated by a modification of the procedure described by Strack *et al.* [4]. Leaves (2 g fr. wt) were homogenized in boiling EtOH (20 ml) and extracted for 15 min in an ultrasonic bath. After centrifugation (10000 *g*; 10 min) the sediment was washed (stirring for 30 min at room temp., unless stated otherwise) with 20 ml of each of the following solvents: MeOH–H_2O (3:7) at 65°, MeOH, Me_2CO, EtOAc and Et_2O, and dried at 60° to constant weight.

Hydrolysis of CWM. For alkaline hydrolysis, the purified CWM (300 mg) was suspended in 1 M aq. NaOH (30 ml) and stirred for 15 hr at room temp. under an atmosphere of Ar. After acidification with conc. HCl the suspension was centrifuged (10000 *g*; 10 min), and the phenolics were extracted from the supernatant into EtOAc (3 × 20 ml). The extract was brought to dryness under reduced pressure and the residue suspended in 0.5 ml MeOH. For enzymic hydrolysis, the purified CWM (300 mg) was washed with 3% $NaHCO_3$ (pH 8.5) and then treated at room temp. under Ar with esterase (Sigma E 3128; 4 U; in aq. Tris–HCl buffer, pH 8; 10 ml; 3 hr), cellulase (Onozuka R-10; 2 mg; in MES–NaOH buffer; pH 8.5; 10 ml; 16 hr) or pectinase (Macerase, Calbiochem, Lot no. 001657; 0.5 mg; otherwise as with cellulase) [31]. Remaining CWM was removed by centrifugation (10000 *g*; 10 min). Following cellulase or pectinase treatment, the supernatant was diluted to 30 ml, supplemented with NaOH to a final concn of 1 M, and digested for 15 hr at room temp. under Ar. After hydrolysis [whether only enzymic (with esterase), or enzymic as well as alkaline], free phenols were extracted from the supernatant as described above. The working-up of the pectinase prepn used to check for contaminating phenols was performed as with CWM; HPLC analysis (see below) demonstrated the presence of ferulic acid as well as vanillin (102 and 690 ng per mg Macerase, respectively).

TLC. Analytical TLC was performed on Silica gel 60 F_{254} (Merck 5554). Solvent systems were (A) CH_2Cl_2–HOAc (4:1) and (B) toluene–diisopropyl ether–HCO_2H (5:4:1). Detection was under UV light at 254 and 366 nm.

HPLC. Samples were separated on an analytical Waters Nova Pak RP-18 column (150 × 3.9 mm) with 2-step gradient elution (1 ml min^{-1}, 1000 psi), changing from 8 to 15% CH_3CN in HOAc–H_2O (0.5:99.5) after 9 min. Peak detection was performed at 265 nm with a Hewlett-Packard High Speed Spectrophotometric Detector 1040 A, serving also for on-line scanning of UV spectra (400–200 nm). Quantitative analysis of peaks (265 nm) was effected with a Hewlett-Packard Integrator 3390 A. Unless stated otherwise, reference phenols were from Fluka.

Isolation of caffeyltartaric acid, quercetin 3-glucuronide and protocatechualdehyde. These were isolated for identification by lobar chromatography on an RP-18 column (Merck 10625 B; 300 × 25 mm). Extracts were chromatographed with H_2O–MeOH–HOAc (94.8:5:0.2) for caffeyltartaric, with H_2O–CH_3CN–HOAc (71.8:28:0.2) for the glucuronide, and with H_2O–CH_3N–HCO_2H (84.8:15:0.2) for the aldehyde. Collection of frs was based on detection at 280 nm, and the identity of compounds was confirmed by UV (caffeyltartaric: max. 240, 325 nm; quercetin glucuronide: max. 254, 350 nm; protocatechualdehyde: max. 231, 280, 311 nm) and ^{1}H NMR (300 MHz, CD_3OD, TMS ($\delta = 0$) as int. standard, coupling constants J in Hz). Caffeyltartaric: 7.66 (1H, *d*, $J = 15.8$, H-α), 7.07 (1H, *d*, $J = 2.0$, H-2), 6.97 (1H, *dd*, $J = 8.1$ and 2.0, H-6), 6.78 (1H, *d*, $J = 8.1$, H-5), 6.32 (1H, *d*, $J = 15.8$, H-β), 5.55 (1H, *br s*, H-2′), 4.76 (1H, *br s*, H-3′); quercetin glucuronide: 7.56 (1H, *dd*, $J = 7.10$ and 1.62, H-5′ or H-6′), 7.54 (1H, *d*, $J = 1.60$, H-2′), 6.77 (1H, *dd*, $J = 7.11$ and 1.79, H-5′ or H-6′), 6.32 (1H, *d*, $J = 2.10$, H-6 or H-8), 6.13 (1H, *d*, $J = 2.10$, H-6 or H-8), 5.26 (1H, *d*, $J = 7.50$, H-1″, β-glycoside), 3.67 (1H, *d*, $J = 9.61$, H-5″), 3.55–3.50 (3H, *m*, H-2″, H-3″ and H-4″); protocatechualdehyde as published [32].

Acknowledgements—This study was supported by the Swiss National Science Foundation (grant no. 31-26961.89 to D.M.R.). The authors thank Prof. W. von Philipsborn, Institute of Organic Chemistry, University of Zürich, for ^{1}H NMR spectra, and Dr H.-P. Ruffner, Institute of Plant Biology of this University, for stimulating discussions.

REFERENCES

1. Harris, P. J. and Hartley, R. D. (1980) *Biochem. Syst. Ecol.* **8**, 153.
2. Hartley, R. D. and Harris, P. J. (1981) *Biochem. Syst. Ecol.* **9**, 189.
3. Ohashi, H., Yamamoto, E., Lewis, N. G. and Towers, G. H. N. (1987) *Phytochemistry* **26**, 1915.
4. Strack, D., Heilemann, J., Mömken, M. and Wray, V. (1988) *Phytochemistry* **27**, 3517.
5. Codignola, A., Verotta, L., Spanu, P., Mattei, M., Scannerini, S. and Bonfante-Fasolo, P. (1989) *New Phytol.* **112**, 221.
6. Van Sumere, C. F. (1989) in *Methods in Plant Biochemistry* (Harborne, J. B., ed.), Vol. 1, pp. 29. Academic Press, London.

7. Hartley, R. D. and Keene, A. S. (1984) *Phytochemistry* **23**, 1305.
8. Fry, S. C. (1989) in *Modern Methods of Plant Analysis*, N. S. Vol. 10, *Plant Fibers* (Linskens, H. F. and Jackson, J. F., eds), p. 12. Springer-Verlag, Berlin.
9. Fry, S. C. (1982) *Biochem. J.* **203**, 493.
10. Sweet, W. J., Morrison, J. C., Labavitch, J. M. and Metthews, M. A. (1990) *Plant Cell Physiol.* **31**, 407.
11. Weber, B. (1993) Ph. D. Thesis. University of Zürich, Switzerland.
12. Singleton, V. L., Timberlake, C. F. and Lea, A. G. H. (1978) *J. Sci. Food Agric.* **29**, 403.
13. Singleton, V. L., Zaya, J. and Tronsdale, E. K. (1986) *Phytochemistry* **25**, 2127.
14. Boursiquot, J.-M., Sapis, J.-C. and Macheix, J.-J. (1986) *C. R. Acad. Sci. Paris* **302**, 177.
15. Egorov, J. A. and Borisova, N. B. (1956) *Chem. Abstr.* **50**, 16699.
16. Grafe, V. (1936) in *Beiträge zur Biologie der Pflanzen* (Winkler, H., ed.), Vol. 24, p. 138. Weidmannsche Buchhandlung, Berlin.
17. Malmberg, A. and Theander, O. (1984) *Swed. J. Agric. Res.* **14**, 119.
18. Harborne, J. B. and Baxter, H. (1993) *Phytochemical Dictionary*. Taylor & Francis, London.
19. Haslam, E. and Lilley, T. H. (1986) in *Studies in Organic Chemistry*, Vol. 23, *Flavonoids and Biflavonoids* (Farkas, L., Gábor, M. and Kallay, F., eds), p. 113. Elsevier, Amsterdam.
20. Carpita, N. C. and Gibeaut, D. M. (1993) *Plant. J.* **3**, 1.
21. Fry, S. C. (1986) *Ann. Rev. Plant Physiol.* **37**, 165.
22. IUB (1992) *Enzyme Nomenclature*, p. 306. Academic Press, San Diego, CA, U.S.A.
23. Doster, M. A. and Schnathorst, W. C. (1985) *Phytopathology* **75**, 318.
24. Langcake, P. and Lovell, P. A. (1980) *Vitis* **19**, 321.
25. Friend, J. (1979) in *Recent Advances in Phytochemistry*, Vol. 12, *Biochemistry of Plant Phenolics* (Swain, T., Harborne, J. B. and Van Sumere, C. F., eds), p. 557. Plenum Press, New York.
26. Rhodes, M. J. C. (1985) in *Annual Proceedings of the Phytochemical Society of Europe*, Vol. 25, *The Biochemistry of Plant Phenolics* (Van Sumere, C. F. and Lea, P. J., eds), p. 99. Clarendon Press, Oxford, U.K.
27. Barz, W. and Köster, J. (1981) in *The Biochemistry of Plants*, Vol. 7, *Secondary Plant Products* (Conn, E. E., ed.), p. 35. Academic Press, New York.
28. Mohan, V. P., Kishore, E., Sugumaran, M. and Vaidyanathan, C. S. (1979) *Plant Sci. Letters* **16**, 627.
29. Vance, T., Kirk, K. and Sherwood, R. T. (1980) *Ann. Rev. Phytopathol.* **18**, 259.
30. Swain, T. (1977) *Ann. Rev. Plant Physiol.* **28**, 479.
31. Ruffner, H. P., Adler, S. and Rast, D. M. (1990) *Phytochemistry* **29**, 2083.
32. Pouchert, C. J. (1983) *The Aldridge Library of NMR Spectra*, Edn 2, Vol. 2, 1f9 A. Aldridge Chemical Co., Milwaukee, WI, U.S.A.