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Synthesis of Di- and Trichlorophenylalanines

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Characterization of some new di- and trichlorinated phenylalanines synthesized via an acetamidomalonate condensation with the appropriate chlorinated benzyl halide is described.

INTRODUCTION

Studies have been made in this laboratory of the metabolic pathway in higher plants leading from L-phenylalanine (L-Phe) to the natural growth-regulating substance, phenylacetic acid (PAA) (*1–5*). In addition to PAA, several synthetic chlorophenylacetic acids have also been shown to be potent plant growth regulators (*6*) and certain of these acids were found to be powerful herbicides (*7*). We decided to prepare a series of ring-substituted chlorophenylalanines to test not only their intrinsic physiological activity but also their ability to serve as potential metabolic precursors for the corresponding chlorophenylacetic acids. We investigated the acetamidomalonate approach (*8*) for chlorophenylalanine synthesis since most of the required chloro-substitution patterns were commercially available as the corresponding benzyl halides, toluenes or benzoic acids, the latter two of which could be readily converted to the appropriate chlorobenzyl halide.

RESULTS AND DISCUSSION

The diethyl acetamidomalonate route used in the synthesis of the chlorophenylalanines (**5a–5l**, Table 2) is outlined in Fig. 1.

The synthetic sequence described in Fig. 1 requires the precursor chloro-substituted benzyl halides **3**. These were prepared in high yield from either the corresponding benzyl alcohols **1** with thionyl chloride (*9*) or the toluenes **2** with N-bromosuccinimide (*10*). The alcohols **1**, including the unknown 2,3,4-trichlorobenzyl analog **1**, were synthesized by reduction of the corresponding benzoic acids (*11*).

Condensation of the benzyl halides with the sodio-derivative of diethyl acetamidomalonate (*12, 13*) produced the chlorobenzyl diethyl acetamidomalonates **4**, which were isolated in pure form in 80–90% yield. These were characterized by

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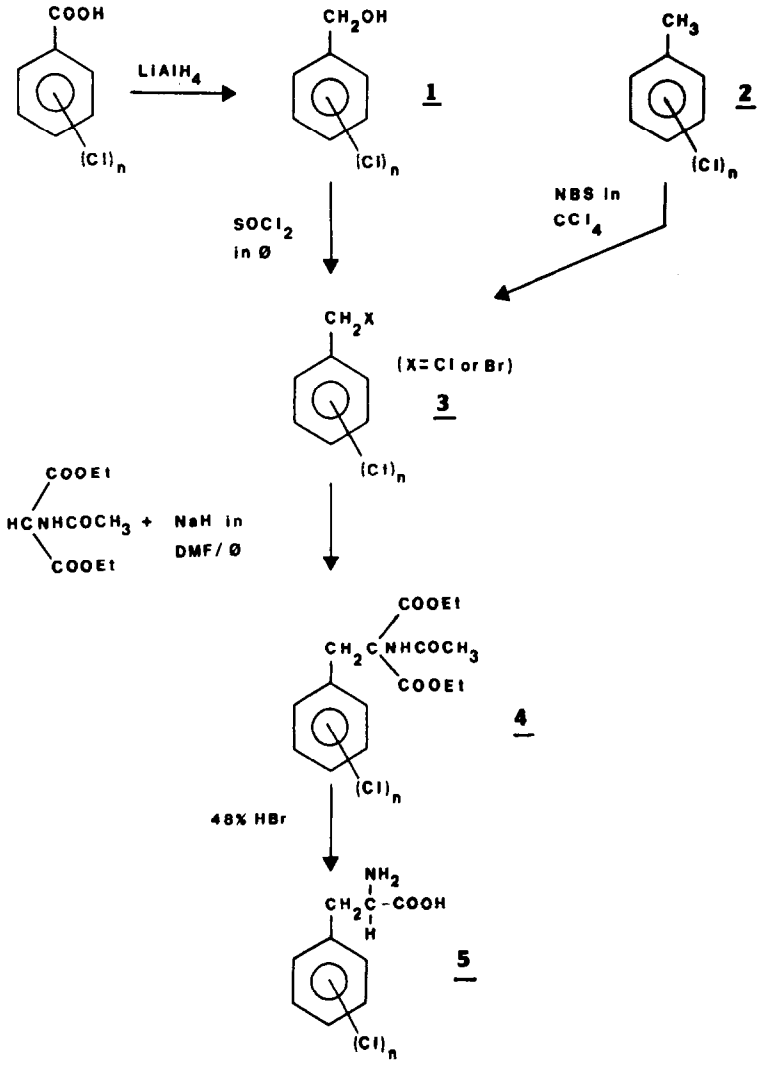

FIG. 1. Acetamidomalonate pathway for chlorophenylalanine synthesis.

melting point and elemental analysis (see Table 1) as well as by IR and ^{1}H NMR spectroscopy.

Hydrolysis and concomitant decarboxylation of **4** in 48% HBr produced the corresponding chlorophenylalanines **5** in isolated yields of 70–90%. Cellulose TLC (butanol : acetic acid : water, 90 : 10 : 29, v/v/v) of both the final recrystallized chlorophenylalanines and their hydrolysates (aqueous phase) (see Table 2) gave single Cd–ninhydrin-positive chromatogram spots (*29*), which gave some indication of the purity of these products and their similarity (in R_f) to D,L-phenylalanine (R_f 0.63). Yields and melting points as well as IR bands in the 800 cm^{-1} region (aromatic CH bending representative of the specific chloro-substitution pattern for the individual chlorophenylalanines) are reported in Table 2.

TABLE 1

Data for the Chloro-Ring-Substituted Acetamidomalonate Derivatives, **4a–4l** (Prepared from Benzyl Halides **3a–3l**)

Compound (substitution pattern)	mp (°C) (lit. values in parentheses[a])	Isolated yield (%)	Microanalysis[b] C	H
4a (2-)	78–80 (94–95)	79	56.10	5.80
4b (3-)	114–116	77	56.15	5.71
4c (4-)	141–142 (143–144)	75	56.04	5.71
4d (2,3-)	127–129	90	51.00	5.23
4e (2,4-)	154–155 (159–160)	87	51.13	5.11
4f (2,5-)	134–135	75	51.16	5.20
4g (2,6-)	126–128	93	51.24	5.15
4h (3,4-)	136–137 (134–136)	90	51.19	5.24
4i (3,5-)	144–146	91	51.16	5.15
4j (2,3,4-)	176–177	83	46.43	4.40
4k (2,3,6-)	128–129	83	46.32	4.49
4l (2,4,5-)	145–146	78	46.30	4.49

[a] See Ref. (*13*).

[b] Calculated for monochloro, $C_{16}H_{20}O_5NCl$, C 56.23, H 5.86%; dichloro, $C_{16}H_{19}O_5NCl_2$, C 51.08, H 5.09%; trichloro, $C_{16}H_{18}O_5NCl_3$, C 46.79, H 4.39%.

The chlorophenylalanines were uniquely characterized by high-resolution ^1H NMR spectroscopy. All derivatives exhibited a broadened doublet of doublets at ~4.1 ppm for the Hα to $-NH_3^+$, –COOH, and the differentiated benzylic $-CH_2$'s as well as a broad singlet at ~6.1 ppm for the $-NH_3^+$, interestingly not exchanged in D_2SO_4. More pertinent, and only discernible at 300 or 500 MHz, were the

TABLE 2

Data for Chloro-Ring-Substituted D,L-Phenylalanines Numbered **5a–5l**

Compound number (and substitution pattern)	mp (°C) (decomp.) (lit. values)	Isolated yield (%)	R_f[a]	IR[b]	^1H NMR Aromatic H	Benzylic H	
5a (2-)	216–219 (226–228[c])	73	0.72	750			
5b (3-)	220–221 (215–216[d])	81	0.72	795			
5c (4-)	228–229 (236–241[c])	80	0.74	820			
5d (2,3-)	238–240	86	0.80	770,710	6.63(1, d), 6.69(1, t), 6.93(1, d)	2.90	3.25
5e (2,4-)	239–241 (237–239[c])	82	0.80	820,870	6.71(1, d), 6.80(1, d), 7.00(1, s)	2.80	3.20
5f (2,5-)	237–238	70	0.77	805,880	6.75(1, s), 6.77(1, d), 6.88(1, d)	2.75	3.10
5g (2,6-)	257–259	75	0.83	780,715	6.65(1, t), 6.73(1, d), 6.90(1, s)	2.88	3.16
5h (3,4-)	213–216 (229–234[c])	83	0.83	810,880	6.75(1, d), 6.88(1, s), 6.92(1, d)	2.70	3.00
5i (3,5-)	229–230	84	0.84	845	6.70(1, s), 6.90(2, s)	2.70	2.95
5j (2,3,4-)	254–257	90	0.88	820,725	7.12(1, d), 7.38(1, d)	3.30	3.65
5k (2,3,6-)	261–263	82	0.87	800,675	6.85(1, d), 6.99(1, d)	3.20	3.35
5l (2,4,5-)	254–255	78	0.83	870	6.92(1, s), 7.10(1, s)	2.80	3.15

[a] R_f values from the TLC on cellulose in BuOH : HOAc : H_2O, 9 : 1 : 2.9 (v/v).

[b] See Ref. (*20*).

[c] See Ref. (*13*).

[d] See Ref. (*16*).

details of the aromatic H splitting patterns which unambiguously define the individual chlorine substitution patterns of each compound. Also the hydrogens on the benzylic C were observed as individual doublets of doublets at ~3.0 ppm since they are differentiated by the chiral center and split by that α-H. It is interesting to note that whenever a chlorine is substituted in an *ortho* position (i.e., 2- or 6-), one of the benzylic protons absorbs at ~2.9 ppm or higher, while if an *ortho*-chlorine is absent, the benzylic proton absorbs at ~2.9 ppm or lower. The only exception is the 2,3,4-trichloro derivative which exhibited all bands 0.2–0.4 ppm further downfield than usual. Values (ppm) for the individual aromatic and benzylic hydrogens are included in Table 2 and some representative 500 MHz spectra are presented in Figs. 2a–2c to illustrate the power of this technique for unambiguously "fingerprinting" these compounds.

TABLE 3

Characteristic MS Fragmentation Patterns Observed for *bis*-PFB Derivatives of Phenylalanine and Chlorophenylalanine

	GC–MS		CI–MS (diethyl ether, probe)	
Phenylalanine derivative	*m/z* (rel. int. %)	Character of fragment ion	*m/z* (rel. int. %)	Character of fragment ion
Unsubstituted (D,L-Phe-PFB)	434 (43)	$(M - \text{benzyl grp})^+$	525 (100)	$(M)^+$
	300 (31)	$(M - \text{COOPFB})^+$	434 (22)	$(M - \text{benzyl grp})^+$
	181 (100)	$(\text{PFB})^+$	300 (25)	$(M - \text{COOPFB})^+$
	91 (10)	$(\text{Benzyl grp})^+$	181 (25)	$(\text{PFB})^+$
Monochloro-substituted (4-Cl-D,L-Phe-PFB)	434 (46)	$(M - \text{Cl benzyl grp})^+$	560 (100)	$(M + H)^+ (Cl^{35})$
	334 (13)	$(M - \text{COOPFB})^+$	562 (35)	$(M + H + 2)^+ (Cl^{37})$
	181 (100)	$(\text{PFB})^+$	434 (35)	$(M - \text{Cl benzyl grp})^+$
	125 (6)	$(\text{Cl-benzyl grp})^+$	380 (45)	$(M + H - \text{PFB})^+$
			334 (15)	$(M - \text{COOPFB})^+$
			254 (11)	$(M + \text{H-Cl benzyl} - \text{PFB})^+$
			181 (38)	$(\text{PFB})^+$
			125 (9)	$(\text{Cl-benzyl grp})^+$
Dichloro-substituted (2,6-Cl_2-D,L-Phe-PFB)	434 (41)	$(M - Cl_2 \text{ benzyl grp})^+$	593 (100)	$(M)^+$
	368 (11)	$(M - \text{COOPFB})^+$	595 (67)	$(M + 2)^+$
	181 (100)	$(\text{PFB})^+$	597 (13)	$(M + 4)^+$
	159 (2.5)	$(Cl_2\text{-benzyl grp})^+$	434 (43)	$(M - Cl_2 \text{ benzyl grp})^+$
			368 (15)	$(M - \text{COOPFB})^+$
			254 (6)	$(M + \text{H-}Cl_2 \text{ benzyl-PFB})^+$
			181 (38)	$(\text{PFB})^+$
			159 (4)	$(Cl_2\text{-benzyl grp})^+$
Trichloro-substituted (2,3,6-Cl_3-D,L-Phe-PFB)	434 (41)	$(M - Cl_3 \text{ benzyl grp})^+$	627 (100)	$(M)^+$
	402 (12)	$(M - \text{COOPFB})^+$	629 (98)	$(M + 2)$
	193 (2.2)	$(Cl_3\text{-benzyl grp})^+$	631 (33)	$(M + 4)^+$
	181 (100)	$(\text{PFB})^+$	633 (2.5)	$(M + 6)^+$
			434 (73)	$(M - Cl_3 \text{ benzyl grp})^+$
			402 (24)	$(M - \text{COOPFB})^+$
			254 (6)	$(M + \text{H-}Cl_3 \text{ benzyl-PFB})^+$
			181 (83)	$(\text{PFB})^+$

GC–MS in the electron impact (EI) mode of the pentafluorobenzyl (PFB) derivative or the trimethylsilyl (TMSi) derivative gave no molecular ion, but did give mass spectral fragmentation ions which were characteristic of *bis*-PFB and -TMSi derivatives, in which one PFB or TMSi moiety presumably esterifies the carboxyl group while the other moiety adds at the amino nitrogen. Similarly, the 2-chloromethyl ester derivative prepared as reported for L-Phe by Thenot and Horning (*14*) gave the fragmentation pattern expected for the 2-chloro-Phe-*N*-dimethylaminomethylene methyl ester product, but again no molecular ion. Thus, chemical ionization mass spectrometry (CI–MS) (*28*) was utilized to analyze the PFB-chlorophenylalanines and confirmed that the PFB derivatives were indeed the *bis* adducts.

Table 3 lists both EI and CI data for unsubstituted phenylalanine as well as representative monochloro-**5c**, dichloro-**5g** and trichloro-**5k** derivatives. These typify the patterns and intensities obtained for all compounds. It is interesting to observe that in the CI spectra, only the monochlorophenylalanines exhibit the expected $M^+ + 1$ peak, whereas the others show only M^+ peaks. This trend was consistent for all samples and although unusual, it is not unprecedented since it depends on the relative ionization efficiencies of probe solvent versus compound (*30*). Additionally, the CI spectra confirm the number of chlorines present based on the relative intensities of the M^+ (or $M^+ + H$) +2, +4, and +6 peaks (*15*).

To our knowledge, the 2,3-, 2,5-, 2,6-, and 3,5-dichlorophenylalanines and the 2,3,4-, 2,3,6-, and 2,4,5,-trichlorophenylalanines have not been reported previously in the literature. The acetamidomalonate approach to chlorophenylalanine synthesis appears to be the method of choice when compared with other reported methods such as the Meerwein (*16*) or azlactone routes (*23–26*). High resolution ^1H NMR and CI–MS can uniquely characterize these compounds.

Studies of the *in vitro* and *in vivo* metabolism of this series of chlorophenylalanines by both crop and weed plants in relation to their potential conversion to the corresponding chlorophenylacetic acids and/or chlorobenzoic acids which may show growth-regulating activity, has been carried out in our laboratory and are reported in several publications (*31–33*).

EXPERIMENTAL

General details. Melting points were obtained on a Buchii SMP-20 melting range apparatus. IR spectra (ν_{max} in cm^{-1}, KBr pellets) were recorded on a Perkin–Elmer 237B grating spectrophotometer. Proton magnetic resonance (^1H NMR) spectra for all intermediate compounds were recorded in ppm (δ) relative to tetramethylsilane (TMS) on a Varian T-60 (60 MHz) instrument. The ^1H NMR spectra for the chlorophenylalanines were recorded at 40°C in δ relative to TMS or sodium tetradeuterotrimethylsilylpropionate (TSP) on a General Electric NT 300 Widebore, 300-MHz instrument, or on a Bruker AM 500, 500-MHz instrument. Spectra were taken in either $CDCl_3$ with 1% TMS or D_2SO_4 with TSP and peak shapes are indicated by s (singlet), d (doublet), q (quartet), dd (doublet of doublets), t (triplet), m (multiplet), and br (broad). Mass spectra (CI with diethyl ether

direct probe or EI with GC introduction) were obtained using a VG-7070E instrument (VG Analytical, Manchester, UK) and a 30-m DB5 megabore column (J & W Scientific). When full spectra were desired the starting temperature was 100 (TMSi derivative) or 200°C (PFB and Me derivative) and after a 2-min isothermal period, a temperature gradient of 10°C min^{-1} was applied. Only diagnostically important ions are reported, with the character of the fragmentation ions in relation to the molecular ion and the intensity relative to the base ion indicated. Microanalyses were obtained from Spang Microanalytical Laboratory, Michigan. Thin-layer chromatography was performed using silica gel G (0.2-mm layers) on glass plates (Merck) or, in the case of the chlorophenylalanines, on cellulose (0.1 mm) precoated on aluminium sheets (EM Laboratories, Inc.). Appropriate chloro-substituted toluenes, benzoic acids, or benzyl halides were available from Aldrich (Milwaukee, WI), Pfaltz and Bauer, Inc., or I.C.N. Pharmaceuticals, Inc. Column chromatography was accomplished using Florosil adsorbent 100–200 mesh (Matheson, Coleman and Bell, Norwood, OH). Pentafluorobenzylbromide (PFBBr), dimethylformamide (DMF)–dimethylacetal (Me-8), and *N,O-bis*(trimethylsilyl) acetamide in pyridine (Tri–Sil BSA-"P") were obtained from Pierce Chemical Co. (Rockford, IL).

Preparation of the benzyl alcohols: **1**. The general method for preparation of the benzyl alcohols from the corresponding carboxylic acids was that reported by Fieser and Fieser (*11*). Lithium aluminum hydride (0.075 mol/0.10 mol of carboxylic acid to be reduced plus a 10% excess) in anhydrous ether (50 ml) was added to a round-bottom flask equipped with a magnetic stirrer, dropping funnel, reflux condenser, and drying tube. With continuous stirring, a solution of the acid in absolute ether (150 ml) was added dropwise such that the ether refluxed gently. After addition was complete, the mixture was refluxed for 1 h or stirred a further 4–6 h at room temperature. The reaction flask was then cooled in ice water. Ethyl acetate followed by ice water was added dropwise to the stirring reaction mixture to decompose the excess hydride. Aluminum salts were redissolved by the addition of 10% H_2SO_4. The reaction mixture was extracted three times with diethyl ether and the combined ether extracts were washed with a 10% aqueous sodium bicarbonate solution, dried, and evaporated. The crude alcohol was passed through a short column of Florosil (5 g adsorbant/g crude alcohol) with MeOH : $CHCl_3$ (5 : 95, v/v) to remove any impurities and yielded ~90% pure alcohol after evaporation of the solvent.

The following known benzyl alcohols were prepared: *2,5-dichloro:* mp 78.5–79°C, lit. mp 78–80°C (*17*); IR, 3250 (–OH stretch), 1465 ($–CH_2$-scissoring), 1025 (–C–O stretch of 1° alcohol), 810, 880 (–CH out of plane bending, 1,2,5-trisubstituted benzene); ^1H NMR, 3.0 (1H, bs, disappears with D_2O, $–CH_2OH$), 4.75 (2H, s, $–CH_2–$), 7.25–7.6 (3H, m, Ar-*H*). *3,5-dichloro:* mp 78–80°C, lit. mp 79–82°C (*17*); IR, 3250 (–OH stretch), 1050 (–C–O stretch, 1° alcohol), 845, 675 (–CH out of plane ring bending, 1,3,5-trisubstituted benzene); ^1H NMR (60 MHz, $CDCl_3$), 2.0 (1H, bs, disappears with D_2O, $–CH_2OH$), 4.7 (2H, s, $–CH_2–$), 7.3 (3H, s, Ar-*H*).

2,3,4-trichlorobenzoic acid was prepared from the commercially available acetophenone by a bromoform reaction (*18*). Bromine (70 g; 24 ml) was added dropwise to a stirred solution (500 ml) of 2 *M* NaOH at 0°C. To this ice-cold hypobro-

mite solution, 2,3,4-trichloroacetophenone (30.4 g, 0.14 mol) in dioxane (75 ml) was added over 10 min. The temperature of the reaction mixture was raised to 50°C over 1 h, and then to 70°C for a further hour with continuous stirring. After cooling, the reaction mixture was washed with CCl_4 (2 × 50 ml) and the alkaline solution was acidified to pH 3 with concentrated HCl. The resulting white solid was filtered, washed with water, dissolved in aqueous bicarbonate, and reprecipitated with HCl. The precipitated solid was then extracted with diethyl ether and dried over anhydrous $MgSO_4$. After removal of the ether and recrystallization from $CHCl_3$, the 2,3,4-trichlorobenzoic acid was obtained: mp 192–193°C, lit. mp 187–188°C (*19*); IR, 3000–2400 (OH stretch), 1700 (C=O stretch); ^{1}H NMR (60 MHz, $CDCl_3$), 7.4 (1H, bs, COO*H*), 7.6–7.9 (2H, dd, Ar-*H*). $LiAlH_4$ reduction of the benzoic acid produced the unknown *2,3,4-trichlorobenzyl alcohol:* mp 99–100°C; IR, 3200 (–OH stretch), 1050 (C–O stretch of 1° alcohol), 820 (C–H out of plane deformation of 1,2,3,4-tetra-substituted benzene); ^{1}H NMR (60 MHz, $CDCl_3$), 3.85 (1H, bs, disappears with D_2O, $-CH_2OH$), 4.8 (2H, s, $-CH_2-$), 7.4 (2H, s, Ar-*H*); (Found: C, 39.91; H, 2.12; $C_7H_5OCl_3$ requires: C, 39.76; H, 2.38%).

Preparation of the benzyl halides: **3**. Preparation of benzyl chlorides used the method of Newman (*9*) in which the alcohol (0.075 mol) was dissolved in anyhydrous benzene (100 ml) and added dropwise over 30 min to a stirring solution of $SOCl_2$ (0.100 mol) in benzene (50 ml) containing one drop of pyridine. After warming to 50–70°C on a water bath for 1–2 h, the mixture was cooled, treated with ice water, extracted three times with ether, dried over $MgSO_4$, and concentrated to yield the benzyl chloride. Filtration through a short column of silica gel (benzene : pet. ether, 1 : 1, v/v) and evaporation of solvent gave the corresponding benzyl chloride as a yellowish liquid (≥95%), essentially pure by ^{1}H NMR (60 MHz, $CDCl_3$), 4.6 (2H, s, $-CH_2-Cl$), 7.3 (Ar-*H*). This method was used to prepare **3f**, **3i**, and **3j**.

Benzyl bromide preparation employed the procedure outlined by Corbin *et al.* (*10*). The toluene (0.030 mol), *N*-bromosuccinimide (0.033 mol), benzoyl peroxide (0.1 g), and dry CCl_4 (80 ml) were placed into a round-bottom flask fitted with a magnetic stirrer and reflux condenser. The bright orange mixture was heated until a white solid (succinimide) was observed floating on the surface of the solvent (usually after 6–8 h of refluxing). If the reaction became vigorously exothermic during refluxing, ice-bath cooling was applied in order to control, but not stop, the reaction. The hot mixture was then suction filtered through a sintered glass funnel and the solid was washed with hot CCl_4 (2 × 50 ml). Removal of the solvent gave essentially quantitative recovery of the material as a pale yellow lachrymatory liquid. ^{1}H NMR (60 MHz, $CDCl_3$), 2.3 (3H, s, unreacted $CH_3-C_6H_5$), ~4.4 (2H, s, $-CH_2Br$), ~7.2 (Ar-*H*). Comparison of the first two peaks indicated that bromination had always proceeded in ~85% yield, and this material was used without further purification. This method was used to prepare **3a**, **3b**, **3d**, and **3l**. All other benzyl halides, i.e., **3c**, **3e**, **3g**, **3h**, and **3k**, were obtained from commercial sources.

Preparation of the chlorobenzyl diethyl acetamidomalonates: **4**. This procedure was a modification (*12*) of the original method of Burckhalter and Stephens (*13*). Sodium hydride (0.03 mol, as a 50% mineral oil dispersion) was freed of mineral

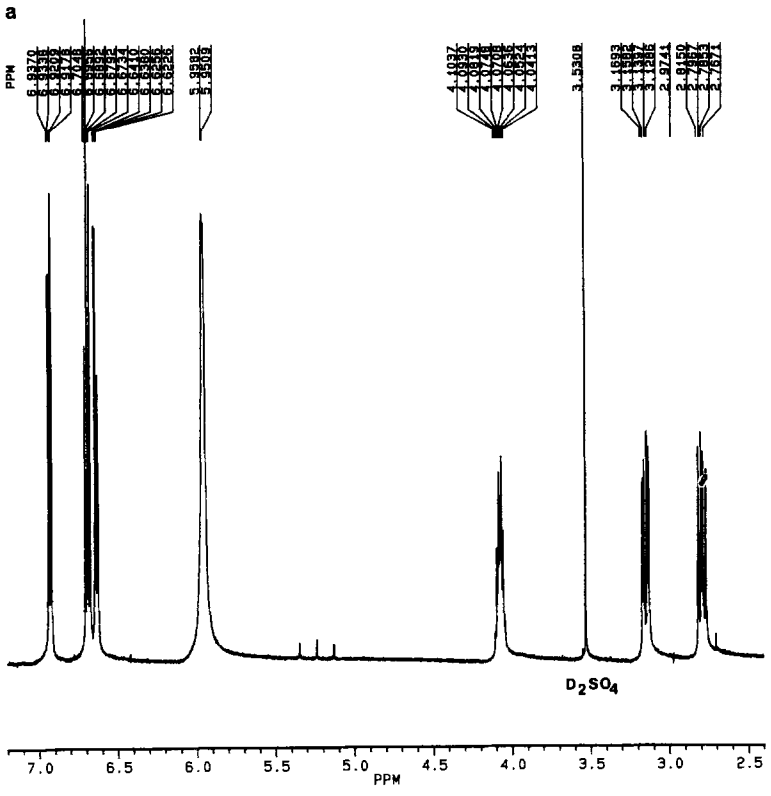

FIG. 2 (a) ^{1}H NMR (500 MHz, D_2SO_4) of 2,3-dichlorophenylalanine, **5d**. (b) ^{1}H NMR (500 MHz, D_2SO_4) of 2,3-dichlorophenylalanine, **5d** expanded "aromatic" region. (c) ^{1}H NMR (500 MHz, D_2SO_4) of 2,6-trichlorophenylalanine, **5g**.

oil by rinsing (2×) with dry petroleum ether and decanting, then suspended in dry DMF : benzene (1 : 1, v/v) (50 ml). Diethyl acetamidomalonate (0.30 mol) in anhydrous DMF : benzene (100 ml) was added dropwise with stirring at room temperature over 1–2 h until evolution of hydrogen was complete. An aliquot of this solution in water produced a pH of ~10. If a solid formed (presumably the sodium salt), it was dissolved by further addition of some DMF. This solution was cooled by an ice bath and the benzyl halide (0.03 mol) in dry DMF (50 ml) was slowly added such that the temperature of the reaction mixture remained below 15°C. This mixture was stirred at room temperature overnight, after which an aliquot in water produced a neutral pH and the condensation was considered complete. The reaction contents were then slowly poured into ice water and extracted with ether (3×), and the combined ether extracts were backwashed with water and then dried over $MgSO_4$. Evaporation of the ether yielded a viscous oil, which, when redissolved in ether and chilled, yielded white crystals in isolated yields of 75–90%. All compounds exhibited some general and characteristic spectral features: IR, 1740 (C=O stretch of ester), 1660 (C=O stretch of amide) as well as bands at 800 (aromatic CH out of plane bending) representative of the appropriate chloro-substitution pattern (20); ^{1}H NMR (60 MHz, $CDCl_3$), ~1.3 (6H, t, 2×-

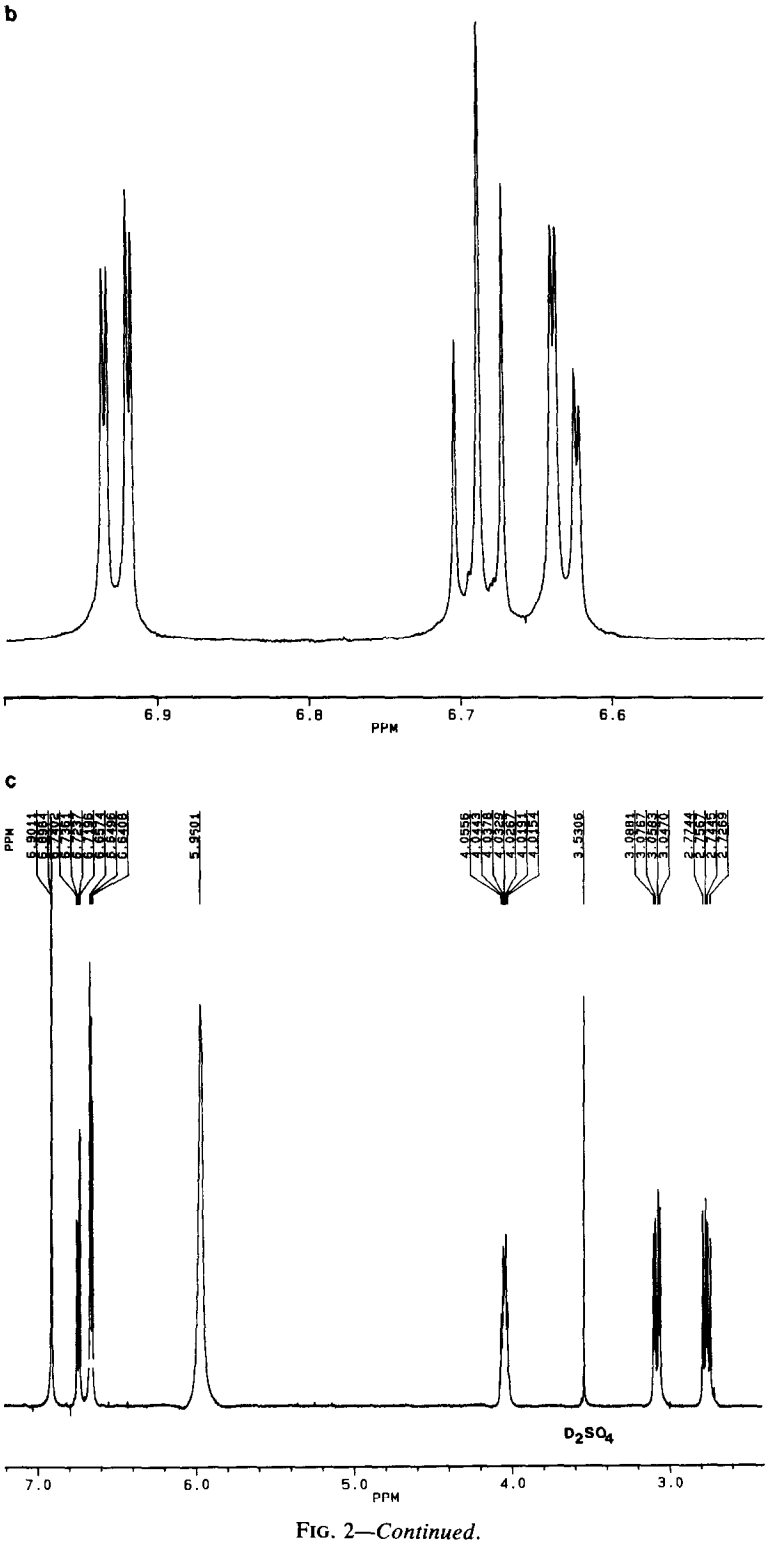

FIG. 2—*Continued.*

$COOCH_2CH_3$), ~2.0 (3H, s, $NHCOCH_3$), ~3.8 (2H, s, $-CH_2-Ar$), ~4.3 (4H, q, $2\times$-$COOCH_2-CH_3$), ~6.7 (1H, bs, NH), ~7.3 (Ar-H). See Table 1 for yields, mp, and analyses of individual compounds.

Preparation of the chloro-substituted D-L-phenylalanines: **5a–5l.** Hydrolysis and decarboxylation of the chlorobenzyl diethyl acetamidomalonate derivatives (**4a–4l**, Fig. 1) by refluxing 48% HBr and isolation of the amino acids was performed essentially as outlined by Burckhalter and Stephens (*13*). The acetamidomalonate derivative (0.015 mol) was refluxed in 48% HBr (50 ml) with stirring for 48–60 h. The reaction mixture was cooled and the insoluble salt which formed was separated by suction filtration and dissolved in a slight excess of NH_4OH. The solution was adjusted to pH 6 with glacial acetic acid and refrigerated overnight to yield the solid whitish amino acid, which was filtered off, washed with ice water (3 × 50 ml) and then washed with acetone. Each amino acid was then recrystallized from aqueous methanol (10%, v/v) or, alternatively, it was dissolved in dilute HCl and reprecipitated by the addition of NH_4OH to pH 6.5. IR spectra of all compounds exhibited features typical of amino acids (*21*); e.g., 3200–2900 (H-bonded NH_2 and OH stretch very broad in solid state), 2500, 2200–2100 (NH_3^+ combination and overtone bands), 1600 (COO–, asymmetrical C–O stretch), 1550–1480 (NH_3^+ asymmetrical bending), 1400 (COO–, symmetrical C=O stretch) as well as bands at 800 (see Table 2) for aromatic CH representative of the specific chloro-substitution pattern (*20*). ^1H NMR spectroscopy (300 or 500 MHz, D_2SO_4) gave characteristic chemical shifts for the expected protonated form of the chlorophenylalanines at 4.1 ± 0.1 (m, 1H, α-H), 6.1 ± 0.1 (bs, 3H, $-NH_3^+$), as well as bands (see Table 2) at ~3.0 (Ar-CH_2) and ~7.0 (Ar-H). Some representative spectra are included as Figs. 2a–2c.

Derivatization of the chlorophenylalanines for GC–MS analysis. The PFB esters were synthesized according to Markham *et al.* (*22*). Anhydrous K_2CO_3 (10 mg) and 1.5 ml of pentafluorobenzyl bromide (PFBBr) in acetone (5 μL PFBBr ml^{-1}) were added to each vial, along with the chlorophenylalanine to be esterified (500 μg). The vials were capped with septum caps and heated at 60°C for 2 h in a Pierce Reactitherm heating module (Pierce Chemical Co.). The solvent was evaporated under nitrogen at 35°C and the residue was dissolved in 1 ml distilled water. The PFB esters were extracted from the aqueous solution with ethyl acetate (3 × 1.5 ml). The ethyl acetate was evaporated to dryness under nitrogen at 35°C.

Silylation was performed with Tri-Sil/BSA Formula "P" (in pyridine) as described by Klebe *et al.* (*27*). The chlorophenylalanine sample to be silylated (5–10 mg) and 1 ml Tri-Sil/BSA (2.5 mEq/ml) were introduced into a screw-cap septum vial. The mixture was shaken vigorously and then warmed to 70°C for 30 min. The completed reaction mixture was then injected directly into the GC–MS.

Methylation was carried out as described by Thenot and Horning (*14*). After 10–50 mg of the chlorophenylalanine was placed in a screw-cap septum vial, 1 ml of methyl-8 (2 mEq/ml in pyridine) was added and the sample was capped and heated to 100°C for 20 min. The reaction was complete as soon as solution of the substrate amino acid was achieved. Aliquots of the reaction mixture were then injected directly into the GC–MS.

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