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Synthesis of α-Aminoacyl[1] Derivatives of Melphalan for Use in Antibody Directed Enzyme Pro-drug Therapy

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This paper is dedicated in memory of Sir Derek Barton

Abstract: L-Alanyl-, D-alanyl-, L-prolyl-, L-pyroglutamyl- and D-phenylalanylmelphalan were synthesized in 8 steps, with the reactive nitrogen mustard moiety formed at the penultimate step. After protection of *p*-nitrophenylalanine with benzyl ester and N-t-butyloxycarbonyl (BOC) groups, the aromatic nitro was reduced to an amine which was reacted with ethylene oxide to give a product with a *bis*(2-hydroxyethyl)amino moiety. After removal of BOC, it was coupled to the relevant N-benzyloxycarbonyl-α-amino acid. Chlorination of hydroxyethyl yielded the *bis*(2-chlorethyl)amino compound. Final removal of protecting groups was by catalytic hydrogenolysis.

A selective method of cancer treatment by cytotoxic drugs remains an elusive goal. Tumor cells are essentially transformed normal cells and as such exhibit very few if any biochemical differences which may be exploited to afford a selective method of treatment. Nevertheless one such difference is the presence of tumor-associated antigens. Monoclonal antibodies with affinity for a wide range of antigens have been successfully raised[2] and are being exploited in the antibody directed enzyme pro-drug therapy (ADEPT) approach to cancer chemotherapy. ADEPT is a two-stage therapy whereby a cytotoxic molecule is selectively generated at the tumor site.[3] A conjugate formed between an antibody and an enzyme is first administered, and becomes localized at the tumor through binding to the tumor-associated antigen. Sufficient time is allowed to elapse for whatever unbound conjugate present to be cleared from the circulation. A latent form of a cytotoxic agent (a pro-drug) is then administered, whereupon the enzyme moiety of the bound conjugate converts the non-cytotoxic pro-drug to the active cytotoxic species. In this way ADEPT overcomes many of the problems associated with direct conjugation of cytotoxic agents to monoclonal antibodies.[3]

The present study reports the synthesis of a series of pro-drugs of melphalan which are activated by aminopeptidase enzymes. Melphalan (**1**), *p-bis*(2-chloroethyl)amino-L-phenylalanine, is a widely used cytotoxic drug which incorporates an active nitrogen mustard moiety linked to L-phenylalanine.[4] Melphalan is actively transported into cells via amino acid transport pathways.[5] It is believed that the free amino group of the phenylalanine moiety is involved in such transport since N-acyl derivatives of melphalan are two orders of magnitude less cytotoxic.[6-8] We reasoned that melphalan could be linked via this amino group to a second α-amino acid, forming a non-cytotoxic pro-drug. For ADEPT, the complementary enzyme will be an aminopeptidase which cleaves the peptide bond with release of free melphalan.[9] Available to us are three bacterially derived aminopeptidases with activities not found in the body, *viz.* D-aminopeptidase,[10] proline iminopeptidase[11] and pyroglutamyl peptidase.[12] These enzymes cleave from the N-terminal of a peptide a D-amino acid, an L-proline and an L-pyroglutamic acid residue respectively. To complement these enzymes three pro-drugs have been synthesized: D-alanylmelphalan (**8b**), L-prolymelphalan (**8c**) and L-pyroglutamylmelphalan (**8d**). D-Phenylalanylmelphalan (**8e**) and L-alanylmelphalan (**8a**) were also synthesized, as possible pro-drug and reference compound respectively. The synthesis of D- and L-alanylmelphalan has earlier been communicated.[13] We present here details of the synthesis of the above five α-aminoacyl[1] derivatives of melphalan, employing a general

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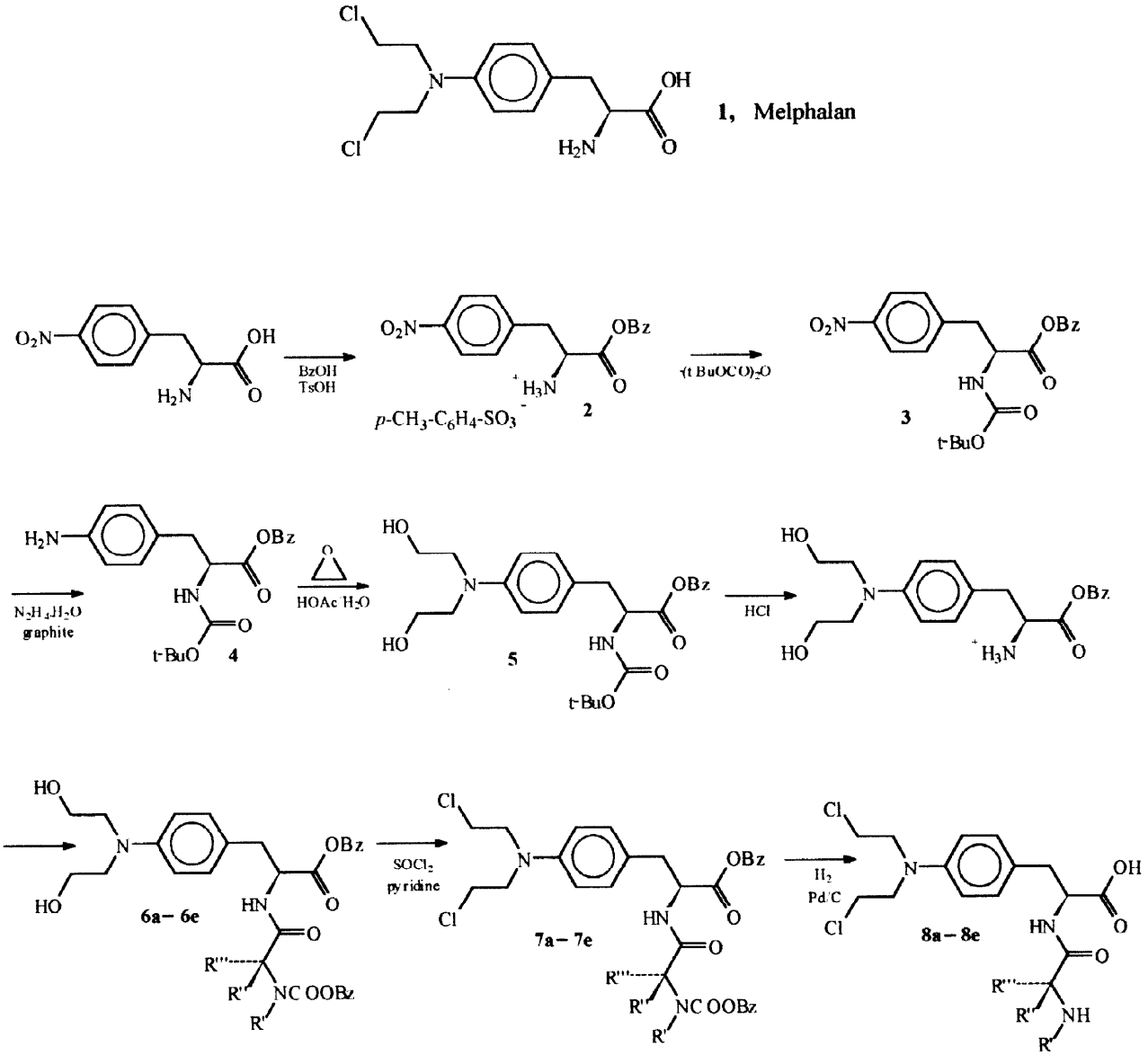
pathway designed to be applicable for the synthesis of unesterified α-aminoacyl[1] and peptide derivatives of melphalan, starting from *p*-nitro-L-phenylalanine, a synthetic precursor of melphalan.

In contrast to our work, previous syntheses of N-acyl, N-aminacyl or N-peptide derivatives of melphalan invariably involved building from melphalan itself or its ester. A number of such derivatives of melphalan ethyl ester were synthesized[14] but no attempt was made to remove the ester group since the nitrogen mustard moiety is highly reactive towards nucleophiles. Hitherto the only known unesterified α-aminoacyl derivative of melphalan is L-valymelphalan which was synthesized via removal of the ethyl ester by strong acid treatment,[14a] the Val-Phe bond being resistant to acid hydrolysis. Ac-Asp-Arg-Val-Tyr-Val-His-Pro-melphalan of doubtful optical purity was prepared via carbodimide coupling to melphalan benzyl ester and hydrogenolysis.[15] Direct acylation of melphalan (unesterified) yielded the following derivatives: acetylmelphalan,[6] phenylacetylmelphalan[8] and *p*-hydroxyphenoxyacetylmelphalan,[7] the last two for use in ADEPT in conjunction with penicillin amidases. β-Alanylmelphalan was synthesized also via direct coupling to melphalan itself.[16]

In our synthesis the reactive nitrogen mustard group was formed near the end of the reaction sequence (Scheme 1). The carboxylic and amino functions of the common stating material *p*-nitro-L-phenylalanine was protected by conversion to the benzyl ester **2**[17] and then the N-t-butyloxycarbonyl derivative **3**.[18] Hydrazine and graphite were then used according to the method of Han *et al.*[19] to reduce the aromatic nitro group, yielding the corresponding amine **4** (76% yield). Through careful monitoring by TLC and addition of extra hydrazine where necessary, hydrazinolysis of the benzyl ester was minimized. Ethylene oxide in 50% aqueous acetic acid was then used to introduce two 2-hydroxyethyl chains to the aromatic amino group, following the method of Bergel and Stock[4] in their original synthesis of melphalan. The product **5** could be obtained pure by preparative TLC and its structure was confirmed by spectroscopy (see Tables 1-3). Nevertheless the crude product produced under optimal conditions (see Experimental) was used directly. The N-t-butyloxycarbonyl protecting group was removed from **5** by acid treatment, immediately prior to coupling with the appropriate N-protected α-amino acid. Coupling was achieved by reacting the deprotected product from **5** in separate reactions with the N-hydroxysuccinimide active esters of N-benzyloxycarbonyl-L- and -D-alanine, L-proline, L-pyroglutamic acid and D-phenylalanine to yield the respective N-benzyloxycarbonyl 'peptide' derivatives **6a** – **6e** (70-76% in 3 steps). The 2-hydroxyethyl chains were then chlorinated with thionyl chloride yielding **7a** – **7e** (50-82%). Finally the required α-aminoacylmelphalan[1] was obtained upon simultaneous removal of the benzyl ester and N-benzyloxycarbonyl groups by hydrogenolysis over palladium-charcoal. L-Alanylmelphalan (**8a**) and D-phenylalanylmelphalan (**8e**) were isolated as the hydrochloride and oxalate respectively (53 and 62%). D-Alanylmelphalan (**8b**) and L-prolylmelphalan (**8c**) were isolated as the free bases (50, 42%). The poor base L-pyroglutamylmelphalan (**8d**) was obtained as a gum (97%). The overall yield in 8 steps for **8a, 8b, 8d** and **8e** was 18-25%, but was lower for L-prolymelphalan partly since its precursors exist in *syn* and *anti* forms (see NMR Tables).

EXPERIMENTAL

Solvents and chemicals used were of AR grade. *p*-Nitrophenylalanine monohydrate, N-benzyloxycarbonyl derivatives of L-proline, L-pyroglutamic acid, D-phenylalanine, and the N-hydroxysuccinimido esters of L-alanine and D-alanine were purchased from Bachem Fine Chemicals. Di-t-butyl carbonate and N,N'-dicyclohexylcarbodiimide were from Aldrich Chemicals. Toluene-*p*-sulfonic acid monohydrate and benzyl alcohol were from AJAX Chemicals. Thionyl chloride was purchased from Merck. Ethylene oxide in a glass ampoule was obtained from Kodak Australia. After the



	R′	R″	R‴
a	H	CH_3	H
b	H	H	CH_3
c	—— CH_2CH_2 ——		H
d	—— $COCH_2$ ——		H
e	H	$CH_2C_6H_5$	H

Scheme 1

ampoule was opened the volatile liquid was stored in a ground-glass stoppered flask in the freezer. Dioxane was dried by distillation from sodium/benzophenone. Chloroform for reactions was washed with water to remove ethanol, then dried by distillation from $CaCl_2$. Benzyl alcohol was distilled at 61-63°C, 1mm Hg. Thionyl chloride was distilled prior to use. Pyridine was distilled and stored over molecular sieves. Other solvents for reactions were distilled and stored under N_2.

Analytical TLC was performed using Merck 60 F_{254} sillica on aluminium-backed plates, and spots were visualized under UV at 254 nm, or following complexation with iodine vapour. Prepartive TLC was carried out using the same silica but on glass-backed plates (0.5 mm thickness). Vacuum chromatography was performed using Merck 60 H TLC-grade silica packed under reduced pressure in a sintered-glass funnel. Melting points were determined on a Reichert hot-stage apparatus and are uncorrected. Optical rotations were measured at 25°C either on a Perkin-Elmer 241 Polarimeter (10 cm cell) or an Optical Activity PolAAr polarimeter (5 cm cell) *using 1% solutions*. Elemental analyses by combustion were performed by Dr B. Pham at the Universtiy of New South Wales. Mass spectra were recorded using a Finnigan/Mat 46 triple-stage quadrupole mass spectrometer with thermal desorption probe. Mass matching by methane chemical ionization was undertaken at the Central Science Laboratory, University of Tasmania. NMR spectroscopy was performed on a Varian Gemini 300 spectrometer. ^1H NMR was carried out at 300 MHz referenced to $SiMe_4$, while ^{13}C NMR at 75 MHz was referenced to the solvent. MS and NMR data not previously reported are given in Tables 1-3.

Table 1. CH_4 Chemical Ionization Mass Spectral Data[a]

	MH^+		$MH^+-C_4H_8$		$MH^+-C_4H_8-CO_2$		MH^+-HCl		MH^+-H_2O		MH^+-nH_2O		Others	
2[b]	301	(100)											138	(75)[c]
3	401	(1)	345	(100)	301	(15)								
4	371	(3)	315	(100)	271	(35)								
5	459	(100)	403	(45)	359	(7)								
6a	564	(100)												
6b	564	(100)												
6c	590	(100)												
6d	604	(100)												
6e	640	(95)											532	(100)[d]
7a[e]	600	(100)					564	(35)						
7b[e]	600	(100)					564	(25)						
7c[e]	626	(100)					590	(30)						
7d[e]	640	(100)					604	(45)						
7e[e]	676	(100)					640	(35)						
8a[b,e]	376	(100)					340	(55)	358	(100)			322	(60)[f]
8b[e]	376	(100)					340	(40)	358	(15)			322	(5)[f]
8c[e]	402	(100)					366	(35)	384	(10)				
8d[e]	416	(100)					380	(60)						
8e[b,e]	452	(60)							434	(100)	416 398	(30) (50)		

[a] Relative abundances given in brackets. Adduct ions $(M+C_2H_5)$ and $(M+C_3H_5)^+$ accompany the quasimolecular ion MH^+.
[b] For salts, data of the acid component not included. [c] $(CH_3C_6H_4NO_2+H)^+$ derived from MH^+ as shown by MS-MS.
[d] Loss of benzyl alcohol appears to be facilitated by presence of the phenylalanine residue. With NH_3 as reagent gas, the same loss also took place, but from the NH_4^+ adduct ion, yielding *m/z* 550 (35%); the quasimolecular ion yields the base peak.
[e] Corresponding ^{37}Cl isotope peak(s) also observed. [f] $MH^+-HCl-H_2O$.

Table 2. 300 MHz ^{1}H NMR Data[a] *(to be continued)*

	Phe Moiety of 'Melphalan-type' Compounds						OBz/CBz[b]		Ala		Pro/Pyro[b]				D-Phe		
	NCH_2 (4 H)	CH_2Cl/OH (4 H)	β (2 H)	α (1 H)	H-3,5 (2 H)	H-2,6 (2 H)	CH_2 (2/2 H)	C_6H_5 (5/5 H)	β (3 H)	α (1 H)	δ (2/0 H)	γ (2 H)	β (2 H)	α (1 H)	β (2 H)	α (1 H)	C_6H_5 (5 H)
4[f]	–	–	2.99 d (*J'* 5.8)	4.57 m	6.56 d (*J'* 8.2)	6.83 d (*J'* 8.2)	5.10, 5.19 ABq (J_{AB} 12.3)	7.3-7.4 m	–	–	–	–	–	–	–	–	–
5[f]	3.55 t (*J'* 4.8)	3.81 t (*J'* 4.8)	2.98 d (*J'* 5.4)	4.56 m	6.58 d (*J'* 8.6)	6.90 d (*J'* 8.6)	5.11, 5.18 ABq (J_{AB} 12.3)	7.3-7.4 m	–	–	–	–	–	–	–	–	–
6a[c,g]	3.51 t (*J'* 5.9)	3.71 t (*J'* 5.9)	2.99 m	4.65 m	6.64 d (*J'* 8.6)	6.98 d (*J'* 8.6)	5.12 m	7.3-7.4 m	1.30 d (7.2)	4.19 m	–	–	–	–	–	–	–
6b[c]	3.45 m	3.65 m	2.88 dd (*J'* 7.5, 13.9) 2.97 dd (*J'* 6.1, 13.9)	4.62 m	6.58 d (*J'* 8.8)	6.90 d (*J'* 8.8)	5.10 m	7.3-7.35 m	1.20 d (7.1)	4.13 m	–	–	–	–	–	–	–
7a	3.58 m, 3.64 m		3.01 d (*J'* 5.6)	4.86 m	6.48 d (*J'* 8.6)	6.87 d (*J'* 8.6)	5.13 m	7.3-7.4 m	1.34 d (7.1)	4.28 m	–	–	–	–	–	–	–
7b	3.67 s		3.01 d (*J'* 5.1)	4.86 m	6.48 d (*J'* 8.6)	6.87 d (*J'* 8.6)	5.16 m	7.3-7.4 m	1.34 d (7.1)	4.30 m	–	–	–	–	–	–	–
8a[d,g]	3.70 s		2.79 dd (9.3, 13.8) 2.97 dd (4.7, 13.8)	4.38 ddd (4.7, 7.9, 9.3)	6.66 d (*J'* 8.7)	7.10 d (*J'* 8.7)	–	–	1.35 d (6.8)	3.82 m	–	–	–	–	–	–	–
8b[d]	3.67 s		2.75 dd (7.3, 13.5) 2.97 dd (4.8, 13.5)	4.15 m	6.59 d (*J'* 8.7)	6.98 d (*J'* 8.7)	–	–	1.10 d (6.8)	3.64 m	–	–	–	–	–	–	–
CBzPro OSu[h]	–	–	–	–	–	–	5.05 - 5.3 m	7.3 - 7.5 m	–	–	3.52 m[l] 3.65 m[l]	2.01 m	2.35 m	4.66 m[l] 4.72 m[l]	–	–	–
6c[d,i]	3.35 m	3.49 m[j]	2.83 m	4.40 m[k]	6.50 d[l] (*J'* 8.6) 6.57 d[l] (*J'* 8.6)	6.92 d[l] (*J'* 8.6) 6.99 d[l] (*J'* 8.6)	5.00 m	7.2 - 7.4 m	–	–	3.35 m	1.71 m	1.71 m 2.04 m	4.24 m[k]	–	–	–
7c[d,i]	3.63 s, 3.69 s		2.86 m	4.44 m	6.55 d[l] (*J'* 8.6) 6.63 d[l] (*J'* 8.6)	6.99 d[l] (*J'* 8.6) 7.05 d[l] (*J'* 8.6)	4.92 m 5.04 m	7.2 - 7.4 m	–	–	3.40 m	1.70 m	1.70 m 2.04 m	4.24 m	–	–	–
8c[e]	3.76 m		3.08 dd (*J'* 7.8, 14.2) 3.21 dd (*J'* 4.7, 14.2)	4.87 m[k]	6.73 d (*J'* 8.4)	7.18 d (*J'* 8.4)	–	–	–	–	3.54 m	2.10 m	2.10 m 2.50 m	4.78 m[k]	–	–	–
CBzPyro OSu[h,k,l]	–		–	–	–	–	5.26, 5.37 ABq (J_{AB} 12.2)	7.3 - 7.4 m	–	–	–	2.3 - 2.8 m		4.98 m	–	–	–

Table 2. 300 MHz ^{1}H NMR Data[a] *(concluded)*

	Phe Moiety of 'Melphalan-type' Compounds						OBz/CBz[b]		Ala		Pro/Pyro[b]				D-Phe		
	NCH_2 (4 H)	CH_2Cl/OH (4 H)	β (2 H)	α (1 H)	H-3,5 (2 H)	H-2,6 (2 H)	CH_2 (2/2 H)	C_6H_5 (5/5 H)	β (3 H)	α (1 H)	δ (2/0 H)	γ (2 H)	β (2 H)	α (1 H)	β (2 H)	α (1 H)	C_6H_5 (5 H)
6d	3.48 t (*J'* 6.0)	3.68 t (*J'* 6.0)	2.85 dd (*J'* 6.4, 13.9) 2.97 dd (*J'* 8.0, 13.9)	4.62 m	6.63 d (*J'* 8.8)	7.00 d (*J'* 8.8)	5.12 m	7.3 - 7.5 m	–	–	–	2.45 m	1.88 m 2.27 m	4.71 m	–	–	–
7d	3.61 m, 3.66 m		2.98 d (*J'* 5.6)	4.87 m	6.53 d (*J'* 8.7)	6.84 d (*J'* 8.7)	5.17 m 5.28 m	7.3 - 7.5 m	–	–	–	2.43 m 2.67 m	2.16 m	4.53 m	–	–	–
8d[c]	3.70 m, 3.76 m		2.93 dd (9.9, 13.5) 3.20 dd (4.5, 13.5)	4.62 m	6.72 d (*J'* 8.8)	7.14 d (*J'8.8*)	–	–	–	–	–	2.27 m 2.41 m	1.96 m 2.27 m	4.17 m	–	–	–
CBz-D-PheOSu	–	–	–	–	–	–	5.10 m	see D-Phe (on right)	–	–	–	–	–	–	3.23 dd (*J'* 6.2, 14.2) 3.33 dd (*J'* 5.8, 14.2)	5.05 m	7.25 - 7.4 m
6e[m]	3.47 t (*J'* 4.5)	3.75 t (*J'* 4.5)	2.94 m 3.07 dd (*J'* 6.8, 13.7)	4.79 m	6.47 d (*J'* 8.6)	6.71 d (*J'*8.6)	5.04 m 5.11 m	see D-Phe	–	–	–	–	–	–	2.81 dd (*J'*5.9, 14.1) 2.94 m	4.44 m	7.1 - 7.4 m
7e[m]	3.56m, 3.65 m		2.96 dd (*J'*5.1, 14.3) 3.06 m	4.82 m	6.42 d (*J'* 8.7)	6.66 d (*J'*8.7)	5.07 m 5.12 m	see D-Phe	–	–	–	–	–	–	2.80 dd (6.3, 13.9) 3.06 m	4.44 m	7.15 - 7.4 m
8e[d,m,n]	3.6 - 3.8 m		2.69 m 2.96 dd (4.7, 13.8)	4.45 m[k]	6.64 d (*J'* 8.6)	7.05 d (*J'*8.7)	–	–	–	–	–	–	–	–	2.69 m 2.88 dd (*J'*4.8, 14.2)	4.03 m[k]	7.1 - 7.3 m

[a] Unless otherwise stated, chemical shift values are in δ downfield from $SiMe_4$ internal standard, as measured in $CDCl_3$ at 300 MHz. The symbols s,d and t may refer to apparent multiplicities. Coupling constants in Hz are given in brackets: *J'* refers to apparent coupling constant. For dilute $CDCl_3$ solutions, signals of CONH, where discerned, appeared at δ 5-7, sometimes showing *J'* of 8-9 Hz. For dilute CD_3SOCD_3 solutions, such signals were found at δ 8-9, with *J'* of 7-8 Hz. Abbreviations: CBz, N-benzyloxycarbonyl; OBz, benzyl ester; OSu, hydroxy-succinimido ester; Pyro, L-pyroglutamate moiety.

[b] OBz and CBz groups often exhibited non-equivalence of benzyl hydrogens, presumably related to orientation of aromatic ring(s).

[c] CD_3OD solvent.

[d] CD_3SOCD_3 solvent.

[e] CD_3COOD solvent.

[f] $(CH_3)_3C$ singlet signals: δ 1.44 for **4**; 1.42 for **5**.

[g] Assignment supported by ^1H - ^{13}C COSY.

[h] CH_2CO multiplet signals: δ 2.83, 2.86 and 2.85 for the hydroxysuccinimido esters of CBzPro,[l] CBzPyro and CBz-D-Phe respectively.

[i] *Syn* and *anti* forms present, in ratio of *ca.* 2:3.

[j] CH_2OH of **6c** assigned by decoupling, and observing collapse of OH signal.

[k] Amino acid α signals distinguished by decoupling.

[l] Data consistent with those reported for a CD_3COOD solution.[23]

[m] Assignments of the partly overlapping signals of the non-equivalent β hydrogens may require reversal.

[n] The sample was an oxalate salt.

Table 3a. ^{13}C NMR Chemical Shifts of Common Intermediates **3** – **5** and of L- and D-Alanyl Compounds[a]

		3	**4**	**5**	**6a**[c]	**6b**[c]	**7a**	**7b**	**8a**[b,d]	**8b**[d]
MelPhe	$N\underline{C}H_2CH_2$	-	-	55.5	54.6	54.7	53.5	53.5	52.1	52.2
	$NCH_2\underline{C}H_2Cl/OH$	-	-	60.7	59.9	59.9	40.5	40.4	41.1	41.1
	β CH_2	38.4	37.4	37.2	37.0	36.9	36.8	36.8	35.4	36.4
	α CH	54.3	54.7	54.8	55.1	54.5	53.5	53.3	54.2	54.7
	3.5 CH	130.2	115.4	112.8	112.7	112.7	112.0	112.0	111.6	111.4
	2,6 CH	123.5	130.2	130.2	130.6	130.5	130.7	130.7	130.2	130.4
	1 C	146.9	125.5	124.1	124.2	124.1	124.3	124.3	125.3	126.5
	4 C	143.8	145.3	146.7	147.7	147.7	145.1	145.2	145.0	144.5
	COOH	-	-	-	-	-	-	-	169.6	170.8
Ala	β CH_3	-	-	-	17.7	17.8	18.8	18.9	18.1	19.0
	α CH	-	-	-	51.2	51.4	50.5	50.6	48.4	48.8
	CO	-	-	-	*174.7	*174.6	*171.3	*171.2	172.5	172.8
OBz/CBz	2,3,4 CH	128.7	128.4	128.5	128.2	128.3	128.1	128.1	-	-
		128.8	128.6	128.5	128.4	128.5	128.3	128.3	-	-
		128.8	128.6	128.6	128.7	128.8	128.6	128.6	-	-
					128.8	128.9	128.6	128.6	-	-
					128.9	128.9	128.7	128.7	-	-
					128.9	129.0	128.7	128.7	-	-
	1 C	134.8	135.4	135.3	136.4	136.4	135.1	135.1	-	-
					137.6	137.6	136.3	136.3	-	-
	CH_2	67.5	67.0	67.1	67.1	66.6	67.0	67.0	-	-
					67.4	67.4	67.3	67.3	-	-
OBz	$\underline{C}OOC$	170.9	172.0	172.1	*172.3	*172.2	*171.9	*171.4	-	-
CBz	OCON	-	-	-	157.6	157.5	155.9	155.9	-	-
BOC	$(\underline{C}H_3)_3C$	28.4	28.4	28.4	-	-	-	-	-	-
	$(CH_3)_3\underline{C}$-O	80.4	79.9	80.0	-	-	-	-	-	-
	OCON	154.9	155.2	155.3	-	-	-	-	-	-

* Assignments within a vertical column may be reversed.

[a] Unless otherwise stated, chemical shifts are in ppm downfield from $SiMe_4$ and referenced to the solvent $CDCl_3$ (77.0 ppm). Recognition of number of attached H on C was by distortionless enhancement by polarization transfer. Assignments are supported by ^1H-^{13}C COSY performed on **6a** and **8a**. Abbreviations: BOC, N-t-butyloxycarbonyl; CBz, N-benzyloxycarbonyl; MelPhe, 'phenylalanine' moiety in 'melphalan-type' compounds.

[b] $(CH_3)_2CHOH$ solvate (see Experimental) had been removed from this sample.

[c] Referenced to the solvent CD_3OD (48.4 ppm).

[d] Referenced to the solvent CD_3SOCD_3 (39.7 ppm).

Table 3b. ^{13}C NMR Chemical Shifts of CBz-Hydroxysuccinimido Esters and of L-Prolyl, L-Pyroglutamyl and D-Phenylalanyl Compounds[a]

		CBz-hydroxysuccinimido esters of:											
		Pro[f]	Pyro[g]	D-Phe	**6c**[d,f]	**6d**	**6e**	**7c**[d,f]	**7d**	**7e**[e]	**8c**[e]	**8d**[c]	**8e**[d,g]
MeIPhe	NCH_2	-	-	-	53.3	53.0	55.3	52.1	53.2	53.5	53.1	**53.8**	**52.0**
	CH_2Cl/OH	-	-	-	58.1	59.7	60.7	41.1	40.2	40.3	40.4	**41.1**	**41.1**
	β CH_2	-	-	-	35.7	36.8	36.8	35.6	36.7	36.7	35.7	**36.6**	***36.2**
	α CH	-	-	-	53.9, 54.3	55.6	56.4	53.8, 54.1	53.8	*56.4	54.5	**54.6**	*53.9
	3,5 CH	-	-	-	111.0	111.3	112.7	111.6	112.6	112.1	112.1	**112.7**	**111.7**
	2,6 CH	-	-	-	129.7	130.1	130.2	130.1	130.7	130.6	130.5	**130.9**	**130.3**
	1 C	-	-	-	123.0	123.6	123.3	124.9	124.8	124.3	124.6	**126.2**	**125.1**
	4 C	-	-	-	146.6	147.0	146.9	144.9	144.6	145.0	145.5	**146.1**	**145.0**
	COOH	-	-	-	-	-	-	-	-	-	*169.6	**174.3**	*168.0
Pro/Pyro[h]	γ CH_2	20.5, 24.7	22.1	-	22.8, 23.6	22.2	-	22.8, 23.6	22.3	-	24.0	**26.1**	-
	β CH_2	30.3, 31.4	30.7	-	29.8, 30.9	31.5	-	29.8, 30.9	31.4	-	30.2	**29.7**	-
	α CH	57.1, 57.4	56.8	-	59.0, 59.5	60.4	-	59.0, 59.5	59.7	-	59.6	**57.4**	-
	δ CH_2	46.4, 46.9	-	-	46.4, 47.0	-	-	46.6, 47.0	-	-	46.9	-	-
	δ CO	-	172.3	-	-	*171.3	-	-	*171.1	-	-	**174.3**	-
Phe	β CH_2	-	-	38.0	-	-	38.6	-	-	38.6	-	-	*37.0
	α CH	-	-	53.0	-	-	53.3	-	-	*53.2	-	-	*53.2
	1 C	-	-	136.0	-	-	*136.4	-	-	*136.4	-	-	134.7
Phe/Pro/Pyro	CO	-	-	-	*171.5	*169.5	*170.6	*171.4	*169.7	*170.5	*176.1	**174.3**	*172.6
Phe/OBz/CBz	2,3,4 CH	128.0, 128.1	128.6	127.6	126.9, 127.4	128.3	127.1	126.8, 127.4	128.1	127.2	-	-	127.0
		128.1, 128.2	128.6	128.2	127.7, 127.9	128.6	128.0	127.7, 127.9	128.5	128.0			128.3
		128.5, 128.5	128.6	128.3	127.9, 128.0	128.6	128.2	128.0, 128.0	128.7	128.3			129.5
				128.6	128.0, 128.1	128.7	128.5	128.1, 128.1	128.7	128.6			
				128.8	128.1, 128.3	128.7	128.5	128,3, 128.3	128.8	128.6			
				129.7	128.3, 128.4	128.7	128.6	128.4, 130.0	128.8	128.7			
							128.6			128.7			
							128.7			128.8			
							129.4			129.4			
OBz/CBz	1 C	136.4, 136.5	134.8	134.4	135.7	135.0	135.2	135.7	135.0	135.1	-	-	-
					137.0	135.1	*136.2	137.0	135.1	*136.2			
	CH_2	67.4, 67.5	68.9	67.4	**65.6**	67.4	67.1	65.6	67.5	67.1	-	-	-
					65.9	68.6	67.2	66.0	68.5	67.3			
OBz	CO	-	-	-	*172.2	*173.7	*171.3	*172.2	*173.2	*171.0	-	-	-
CBz	CO	154.1, 154.6	150.2	155.4	**153.7**	151.6	155.9	153.8	151.7	155.9	-	-	-
OSu[h]	NCO,OCO	168.4	167.2	167.6	-	-	-	-	-	-	-	-	-
		168.7	168.5	168.7	-	-	-	-	-	-	-	-	-
	CH_2	25.7	25.6	25.6	-	-	-	-	-	-	-	-	-

For footnotes a-d, see Table 3a.

* Assignments of like signals within a vertical column may be reversed.

[e] Referenced to the solvent CD_3COOD (177.3 ppm for CO).

[f] *Syn* and *anti* forms both present.

[g] Sample is an oxalate salt with signal at 164.3 ppm.

[h] Further abbreviations: OSu, hydroxysuccimido ester; Pyro, L-pyroglutamyl.

N-t-Butyloxycarbonyl-p-*nitro-L-phenylalanine benzyl ester* (**3**). *p*-Nitro-L-phenylalanine benzyl ester (**2**), prepared as the toluene-*p*-sulfonate following a recent procedure,[20] has physical[17] and ^{1}H and ^{13}C NMR data[20] indentical to the literature values. It was converted to the N-t-butyloxycarbonyl (BOC) derivative by the method used by Tarbell *et al.*[21] to synthesize BOC-glycine ethyl ester. Thus **2** (10.0 g, 21.2 mmol) was suspended in cholroform (120 mL) and $NaHCO_3$ (1.78 g, 21.2 mmol) and NaCl (2 g) in water (90 mL) were added, followed by di-t-butyl carbonate (9.24 g, 42.4 mmol) in chloroform (10 mL). After the mixture was refluxed for 2 h, the aqueous layer was extracted with chloroform and the chloroform solutions washed with saturated aqueous NaCl, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was crystallized from aqueous methanol and dried under vacuum over P_2O_5 to give **3** as needles (8.1 g, 94%), mp 83-84°C, $[\alpha]_D$ – 18.3° (ethanol) (lit 82-83 °C, $[\alpha]_D$ – 19.2° (*c* 1, ethanol)[18]); the ^{1}H NMR data were identical to those reported.[18]

p-*Amino-N-t-butyloxycarbonyl-L-phenylalanine benzyl ester* (**4**). To **3** (8.0 g, 20 mmol) dissolved in dry dioxane (30 mL) was added powdered graphite (12 g) and hydrazine hydrate (2.0 mL, 40 mmol). The thick, black mixture was heated with stirring at 105°C under N_2. After 3 h it was analysed by TLC. If unreacted starting material was present, additional hydrazine hydrate (1.0 mL portions) was added and the mixture heated for further 1 h periods. Graphite was filtrated and washed repeatedly with ethanol. The residue obtained on removal of solvents *in vacuo* was crystallized from aqueous methanol (with removal of hydrazide by-products) to give plates of **4** (5.7 g, 76%), mp 89-90°C, $[\alpha]_D$ – 6.6° (ethanol) (Found: C, 68.15; H, 7.28; N, 7.64%. $C_{21}H_{26}N_2O_4$ requires C, 68.09; H, 7.07; N, 7.56%).

N-t-butyloxycarbony-p-bis*(2-hydroxyethyl)amino-L-phenylalanine benzyl ester* (**5**). A solution of **4** (2.0 g, 5.4 mmol) in 1:1 water:acetic acid (54 mL) was cooled in ice, and ethylene oxide (6.0 mL) was added. The mixture was stirred at room temperature for 24 h in a stopped flask protected from light. The yellow solution was poured into water (200 mL), and $NaHCO_3$ (0.56 g, 6.6 mmol) was added slowly with stirring. The gummy precipitate was extracted with ethyl acetate. Upon drying with $MgSO_4$ and removal of solvents *in vacuo*, an orange gum was obtained which, being of fair purity by TLC analysis, was used in subsequent reactions. Purification of a portion by preparative TLC (ethyl acetate) produced **5** as a gum, $[\alpha]_D$ – 4.3° (ethanol) (Found: MH^+ by CH_4 CI MS, 549.250. $C_{25}H_{34}N_2O_6$ requires MH^+ 459.249).

Removal of the t- butyloxycarbonyl protecting group from **5**. A solution of **5** (from 5.4 mmol of **4**) in 4 M HCl in 3:2 water:dioxane (20 mL) was stirred for 2 h unstoppered (to allow release of CO_2). The reaction mixture, basified by addition of saturated aqueous Na_2CO_3, was extracted with dichloromethane. The solution, dried with $MgSO_4$, was evaporated to give the deprotected product as an orange gum which was used immediately.

N-(N-Benzyloxycarbonyl-L-alanyl)-p-bis*(2-hydroxyethyl)amino-L-phenylalanine benzyl ester* (**6a**). To crude deprotected product (above) in dry dioxane (20 mL) was added with stirring N-benzyloxycarbonyl-L-alanine N-hydroxysuccinimido ester (1.70 g, 5.4 mmol). After 90 min, dioxane was removed *in vacuo* and the remaining orange gum redissolved in ethyl acetate and washed with saturated aqueous Na_2CO_3 to remove N-hydroxysuccinimide. The aqueous extracts were washed with ethyl acetate, and the combined ethyl acetate solutions dried ($MgSO_4$) and evaporated *in vacuo* to give an orange gum which was purified by vacuum chromatography over silica with elution by ethyl acetate to give **6a** as needles from ethyl acetate (2.3 g, 76% in 3 steps), mp 124-125°C, $[\alpha]_D$ – 18.3° (methanol) (Found: C, 66.15; H, 6.78; N, 7.56%. $C_{31}H_{37}N_3O_7$ requires C, 66.06; H, 6.62; N, 7.46%).

N-(N-Benzyloxycarbonyl-D-alanyl)-p-bis*(2-hydroxyethyl)amino-L-phenylalanine benzyl ester* (**6b**). To a dry dioxane solution (20 mL) of the crude deprotected product from **5** (derived from 5.4 mmol of **4**) N-benzyloxycarbonyl-D-

alanine N-hydroxysuccinimido ester (1.70 g, 5.4 mmol) was likewise added with stirring. The reaction and work-up were carried out as described above. Upon vacuum chromatography with elution by ethyl acetate, **6b** was obtained as needles from ethyl acetate (2.2g, 72% in 3 steps), mp 111-112°C, $[\alpha]_D$ + 10.3° (methanol) (Found: C, 66.35; H, 6.76; N, 7.57%. $C_{31}H_{37}N_3O_7$ requires C, 66.06; H, 6.62; N, 7.46%).

N-(N-Benzyloxycarbonyl-L-prolyl)-p-bis*(2-hydroxyethyl)amino-L-phenylalanine benzyl ester* (**6c**). The crude deprotected product from **5** was likewise reacted with N-benzyloxycarbonyl-L-proline N-hydroxysuccinimido ester[22] (1.87g, 5.4 mmol), prepared following the literature method. The reaction, work-up and purification were as for **6a**, yielding *syn* and *anti* forms of **6c** as needles from ethyl acetate – light petroleum (2.4 g, 73% in 3 steps), mp 55-75°C, $[\alpha]_D$ – 15.8° (dioxane) (Found: C, 65.40; H, 6.97; N, 6.86%. $C_{33}H_{39}N_3O_7.H_2O$ requires C, 65.22; H, 6.80; N, 6.91%).

N-(N-Benzyloxycarbonyl-L-pyroglutamyl)-p-bis*(2-hydroxyethyl)amino-L-phenylalanine benzyl ester* (**6d**). The crude deprotected product from **5** was likewise reacted with N-benzyloxycarbonyl-L-pyroglutamic acid N-hydroxysuccinimido ester[23,24] (1.95g, 5.4 mmol), and worked up and purified to give **6d** as needles from ethyl acetate (2.2 g, 72%), mp 138-140°, $[\alpha]_D$ + 25.5° (dioxane) (Found: C, 65.42; H, 6.39; N, 7.10%. $C_{33}H_{37}N_3O_8$ requires C, 65.66; H, 6.18; N, 6.96%).

N-(N-Benzyloxycarbonyl-D-phenylalanyl)-p-bis*(2-hydroxyethyl)amino-L-phenylalanine benzyl ester* (**6e**). The crude deprotected product from **5** was likewise reacted with N-benzyloxycarbonyl-D-phenylalanine N-hydroxysuccinimido ester[22,25] (2.14 g, 5.4 mmol), and worked up and purified to give **6e** as needles from ethyl acetate (2.40g, 70% in 3 steps), mp 140-142°C, $[\alpha]_D$ + 3.0° (dioxane) (Found: C, 69.31; H, 6.70; N, 6.77%. $C_{37}H_{41}N_3O_7$ requires C, 69.47; H, 6.46; N, 6.57%).

Chlorination of the bis*(2-hydroxyethyl)amino group of* **6a - 6e** *to yield the nitrogen mustards* **7a - 7e**. Individually **6a - 6e** (1.0 g) was dissolved in dry, ethanol-free chloroform (20 mL). The solution was cooled in an ice-bath before thionyl chloride (3 mL) and pyridine (0.6 mL) were added. The ice-bath was removed and the reaction mixture stirred at room temperature for 1 h, then at 40-45°C for 2 h. The chloroform and excess thionyl chloride were removed *in vacuo*. The black residue was dissolved in dichloromethane and washed twice with saturated aqueous NaCl to remove pyridinium hydrochloride. Combined aqueous washes were extracted with dichloromethane, and the combined dichloromethane solutions were dried ($MgSO_4$) and evaporated. Products were isolated from the resulting black gum by vacuum chromatography over TLC-grade silica using, as eluting solvent, ethyl acetate in dichloromethane in the following ratios: **7a** and **7b**, 1:10; **7c**, 3:20; **7d**, 1:5; **7e**, 1:20.

N-(N-Benzyloxycarbonyl-L-alanyl)-p-bis*(2-chloroethyl)amino-L-phenylalanine benzyl ester* (**7a**). Chlorination of **6a** (1.0 g, 7.78 mmol) was carried out as described above to give the nitrogen mustard **7a** as needles from 2-propanol (0.70 g, 66%), mp 128-129°C, $[\alpha]_D$ – 14.0° (methanol) (Found: C, 62.05; H, 6.10; N, 6.77%. $C_{31}H_{35}Cl_2N_3O_5$ requires C, 62.00; H, 5.87; N, 7.00%).

N-(N-Benzyloxycarbonyl-D-alanyl)-p-bis*(2-chloroethyl)amino-L-phenylalanine benzyl ester* (**7b**). Chlorination of **6b** (1.0 g, 1.78 mmol) was likewise carried out to give the nitrogen mustard **7b** as needles from 2-propanol (0.75 g, 71%), mp 128-129°C, $[\alpha]_D$ + 6.3° (methanol) (Found: C, 62.21; H, 6.12; N, 6.92%. $C_{31}H_{35}Cl_2N_3O_5$ requires C, 62.00; H, 5.87; N, 7.00%).

N-(N-Benzyloxycarbonyl-L-prolyl)-p-bis*(2-chloroethyl)amino-L-phenylalanine benzyl ester* (**7c**). Likewise **6c** (1.0

g, 1.65 mmol) was chlorinated to give **7c** as a white solid from diethyl ether at 4°C (0.55 g, 53%), mp 65-75°C, $[\alpha]_D$ – 12.7° (dioxane) (Found: C, 63.28; H, 6.18; N, 6.65%. $C_{33}H_{37}Cl_2N_3O_5$ requires C, 63.26; H, 5.95; N, 6.71%).

N-(N-Benzyloxycarbonyl-L-pyroglutamyl)-p-bis*(2-chloroethyl)amino-L-phenylalanine benzyl ester* (**7d**). In the same way **6d** (1.0 g, 1.66 mmol) yielded **7d** as needles from 2-propanol (0.53 g, 50%), mp 143-145°C, $[\alpha]_D$ + 23.7° (dioxane) (Found C, 61.84; H, 5.76; N, 6.50%. $C_{33}H_{35}Cl_2N_3O_6$ requires C, 61.88; H, 5.51; N, 6.56%).

N-(N-Benzyloxycarbonyl-D-phenylalanyl)-p-bis*(2-chloroethyl)amino-L-phenylalanine benzyl ester* (**7e**). In the same way **6e** (1.0 g, 1.56 mmol) yielded **7e** as needles from ethanol (0.87 g, 82%), mp 180-181°C, $[\alpha]_D$ + 0.30° (dioxane) (Found: C, 65.64; H, 6.03; N, 6.26%. $C_{37}H_{39}Cl_2N_3O_5$ requires C, 65.68; H, 5.81; N, 6.21%).

L-Alanylmelphalan (**8a**). The N-benzyloxycarbonyl benzyl ester **7a** (100 mg, 0.167 mmol) was dissolved in methanol (5 mL), and 1.0 M aqueous HCl (167 μL, 0.167 mmol)[26] and 10% palladium-on-charcoal (20 mg) were added. Hydrogen was then bubbled through the mixture with stirring. After 2 h at room temperature, the catalyst was removed by filtration, and the solvent evaporated *in vacuo*. The white residue was crystallized from 2-propanol to yield needles of the solvated hydrochloride (see below) of L-alanylmelphalan (**8a**) (40 mg, 53%), mp 126-128°C, $[\alpha]_D$ + 7.9° (M HCl) (Found: C, 46.40; H, 6.68; N, 8.94%. $C_{16}H_{23}Cl_2N_3O_3$,HCl.½C_3H_8O.½H_2O requires C, 46.52; H, 6.47; N, 9.30%). Following drying at 95°C under 0.5 mm Hg pressure for 5 h, mp and $[\alpha]_D$ were unchanged (Found: C, 46.66; H, 6.10; N, 10.23%. $C_{16}H_{23}Cl_2N_3O_3$,HCl requires C, 46.56; H, 5.86; N, 10.18%).

D-Alanylmelphalan (**8b**). The N-benzyloxycarbonyl benzyl ester **7b** (100 mg, 0.167 mmol) was likewise subjected to hydrogenolysis in methanol (10 mL) over palladium-charcoal, but in the absence of added acid. After removal of catalyst, methanol was evaporated slowly *in vacuo* with chilling. D-Alanylmelphalan (**8b**) precipitated out as a white powder (32 mg, 50%), mp 209-211°C, $[\alpha]_D$ + 12.2° (M HCl) (Found: C, 50.72; H, 6.40; N, 10.98%. $C_{16}H_{23}Cl_2N_3O_3$ requires C, 51.07; H, 6.16; N, 11.17%).

L-Prolylmelphalan (**8c**). The N-benzyloxycarbonyl benzyl ester **7c** (100 mg, 0.160 mmol) in methanol (5 mL) was hydrogenated as for the preparation of **8b** above . The white residue obtained upon removal of methanol (27 mg, 42%) was pure by TLC. It was crystallized from dimethyl sulfoxide, washed with ethanol and dried under vacuum to yield L-prolymelphalan (**8c**) as plates (12 mg), mp 175-177°C, $[\alpha]_D$ – 25.2° (M HCl) (Found: C, 51.42; H, 6.47; N, 9.72%. $C_{18}H_{25}Cl_2N_3O_3.H_2O$ requires C, 51.43; H, 6.47; N, 10.00%).

L-Pyroglutamylmelphalan (**8d**). Hydrogenation of **7d** (100 mg 0.156 mmol) in methanol (5 mL) was carried out as above. During the 2 h period the solid starting material dissolved. The catalyst and methanol were removed to yield L-pyroglutamylmelphalan (**8d**) as a clear gum (64 mg, 97%), $[\alpha]_D$ + 27.2° (methanol) (Found: MH^+ by CH_4CI MS, 416.114. $C_{18}H_{23}Cl_2N_3O_4$ requires MH^+ 416.114).

D-Phenylalanylmelphalan (**8e**). **7e** (100 mg, 0.148 mmol) was hydrogenated as for the preparation of **8a** but in the presence of 1.0 M aqueous oxalic acid (148 μL, 0.148 mmol). The oxalate of D-phenylalanylmelphalan (**8e**) crystallized from methanol - diethyl ether as needles (50 mg, 62%), mp 180°C (dec), $[\alpha]_D$ + 13.2° (M HCl) (Found: C, 53.21; H, 5.66; N, 7.75%. $C_{22}H_{27}Cl_2N_3O_3,C_2H_2O_4$ requires C, 53.15; H, 5.39; N, 7.75%).

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24. Our sample prepared by the method of Anderson *et al.*[22] has $[\alpha]_D$ which matched the literature data but has the higher mp of 136–137°C (lit[22] 131-132°C).
25. Our sample prepared by the method of Anderson *et al.*[22] has mp which matched the literature data but has higher $[\alpha]_D$ of + 17.2° (*c 2,* dioxane) (lit[22] + 15.9° (*c 2,* dioxane)).
26. If HCl was in excess, esterification during crystallization with 2-propanol occurred.