N-Methylamino Acids in Peptide Synthesis. II. A New Synthesis of *N*-Benzyloxycarbonyl,*N*-Methylamino Acids[1]

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Reaction of *N*-benzyloxycarbonyl derivatives of aliphatic amino acids, and threonine, aspartic, and glutamic acids whose side-chains were protected with the *t*-butyl group, gave the corresponding *N*-methylamino acid derivatives in good yields. The methionine derivative could be obtained by using only one mol of methyl iodide. Derivatives of threonine, and aspartic and glutamic acids whose side-chains were not protected could not be methylated. Analysis of the crude products of methylation in three cases showed that they contained 0–1% of racemized material.

La réaction des dérivés *N*-benzyloxycarbonyle d'acides aminés aliphatiques, de la thréonine, et des acides aspartique et glutamique dont les chaînes latérales furent bloquées avec le groupe *t*-butyle, a conduit aux dérivés *N*-méthylés correspondants des acides aminés avec d'excellents rendements. Le dérivé de la méthionine a pu être obtenu en utilisant seulement une mole d'iodure de méthyle. Les dérivés de la thréonine et des acides aspartique et glutamique dont les chaînes latérales ne sont pas protégées, n'ont pu être méthylés. L'analyse des produits bruts de la méthylation a montré que dans trois cas, ceux-ci contenaient de 0 à 1% de produits racémisés. [Traduit par le journal]

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The methylation of various *N*-protected amino acids using sodium hydride – methyl iodide in tetrahydrofuran (THF)–dimethylformamide (10:1) under reflux gives the *N*-methylated, methyl ester (1). Similar products have been obtained using silver oxide – methyl iodide in dimethylformamide at room temperature (2). Other methods of *N*-methylation (3–5) used for the permethylation of peptides for sequence analysis by mass spectrometry invariably give methyl esters of the carboxyl functions.

In order to obtain a derivative suitable for peptide synthesis by this approach, it is necessary either to saponify the methyl ester (1, 2) or to remove the amino protecting group (1). It has been shown in this laboratory that saponification of methyl esters of *N*-benzyloxycarbonyl,*N*-methylamino acids or peptides can cause racemization (6). For example, saponification of Z-MeIle-OMe[2] gave 12% of the *allo*-isomer, and saponification of Z-Ala-MeLeu-OMe gave 11% of the L,D-isomer. To circumvent this problem, the methylation step should not esterify the carboxyl group.

In a continuing investigation of the conditions for *N*-methylation, it was found that *N*-benzyloxycarbonylamino acids could be selectively *N*-methylated using sodium hydride (3 mol) and methyl iodide (8 mol) in neat THF at room temperature, without ester formation (8).[3] This observation is supported by the fact that the sodium salt of Z-Ala was esterified only very slowly under these conditions (33% ester formation in 5 days). *N*-Methylation is virtually complete in 24 h; for the sterically hindered Z-*a*Ile, only 0.7% of the unreacted amino acid was detected on analysis of the crude product of methylation.

We have prepared several *N*-benzyloxycarbonyl,*N*-methylamino acids by this method. The properties of the crystalline derivatives obtained are given in Table 1. Z-MeAsp and Z-MeGlu were obtained by methylation of the ω-*t*-butyl esters followed by treatment with trifluoroacetic acid since it was found that Z-Asp and Z-Glu were completely resistant to methylation (the reaction was incomplete even

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[2]The abbreviations for the amino-acid and peptide derivatives are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (7). The amino-acid symbols represent the L-isomer.

[3]It was found, in addition, that methylation of Z-Tyr or Z-Tyr(Z) under these conditions gave Z-MeTyr(Me), and that MeTyr could be obtained by methylation of Z-Tyr(Bzl) followed by hydrogenation (8*a*).

TABLE 1. Properties of *N*-benzyloxycarbonyl,*N*-methylamino acids*

Amino acid	Yield (%)	M.p. (°C)	$[\alpha]_D^{27}$† (°)	Formula	Analysis Calculated C	H	N	Found C	H	N
Leu‡	77	73–74	−23.0	$C_{15}H_{21}NO_4$	64.5	7.6	5.0	64.5	7.5	5.3
Val§	90	68–69	−84.8	$C_{14}H_{19}NO_4$	63.4	7.2	5.3	63.3	7.0	5.4
Ile‖	83	55–56	−68.1	$C_{15}H_{21}NO_5$	64.5	7.6	5.0	64.7	7.7	5.2
D-*a*Ile¶	80	86–88	+68.0	$C_{15}H_{21}NO_5$	64.5	7.6	5.0	64.6	7.9	5.3
Asp	59	127–129	−36.6	$C_{13}H_{15}NO_6$	55.5	5.4	5.0	55.9	5.4	4.9
Glu	43	79–80	−25.7	$C_{14}H_{17}NO_6$	56.9	5.8	4.7	56.7	5.5	4.9

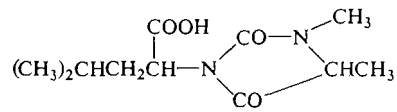
*Compounds crystallized by trituration with light petroleum, and recrystallized from ether – light petroleum; Z-MeAsp, crystallized from water and then ether – light petroleum; Z-MeGlu, crystallized twice from water.
†*c*, 1 in ethanol.
‡Literature m.p. 73–74°, $[\alpha]_D^{25}$ −26.0° (*c*, 2 in dimethylformamide) (1*b*).
§Literature m.p. 69–70°, $[\alpha]_D^{24}$ −85.8° (9).
‖Literature m.p. 63–64°, $[\alpha]_D^{26}$ −72.4° (10). Analysis of a sample, deprotected by hydrogenation in 80% aqueous acetic acid before crystallization, showed a 1.1% content of MeaIle.
¶Analysis of a sample as above showed no MeIle and a 0.7% content of *a*Ile.

in dimethylformamide). The products obtained from the di-*t*-butyl esters were also pure, but they had lower optical rotations, indicating that partial racemization had occurred. We suggest that the α-carboxyl group when ionized provides some protection against racemization by preventing ionization at the α-carbon atom. Z-Asp(OMe)-OMe after methylation and saponification gave a product with an even lower rotation, probably due to racemization taking place during saponification (6).

Methylation of Z-Thr gave a mixture of *N*-methylated (~60%) and *O*-methylated (~25%) products, together with some unchanged starting material. Z-Thr(Bu^t), however, gave the oily *N*-methylated derivative in good yield, which on hydrogenation gave crystalline MeThr(Bu^t). Methylation of Z-Met gave a water-soluble product, probably the dimethylsulfonium iodide derivative. However, by restricting the amount of methyl iodide to 1 mol (11, 12) it was possible to methylate without sulfonium salt formation, giving Z-MeMet as an oil. Removal of the benzyloxycarbonyl group with hydrogen bromide in acetic acid gave a product containing 3.4% of methionine. Z-Trp gave on methylation the α-*N*,ω-*N*-dimethyl derivative as an oil.

Attempts to crystallize Z-MeAsp(OBu^t), Z-MeGlu(OBu^t), and Z-MeThr(Bu^t) as their dicyclohexylamine salts revealed that these were soluble in light petroleum. This property enabled the removal of small amounts of unreacted benzyloxycarbonylamino acid derivatives from the reaction products as the dicyclohexylamine salts which are insoluble in this solvent. Thus benzyloxycarbonylamino acids with suitably protected carboxylic acid and hydroxyl side-chains can also be *N*-methylated to give directly a derivative suitable for peptide synthesis.

A possible strategy for the synthesis of *N*-methylated peptides is methylation of the *N*-protected peptide (1*a*). The methylation of Z-Ala-Leu gave a 58% yield (by weight) of an oil, whose n.m.r. spectrum showed no peaks for the benzyl group. Formation of the hydantoin **1**



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would explain this result. This could have arisen from prior ionization of the amide nitrogen followed by cyclization and loss of benzyl alcohol. That this had occurred is supported by the fact that Z-Ala-MeLeu on methylation yielded the *N*,*N*′-dimethyl derivative in good yield.

The method introduced in this laboratory (13) for determination of the optical purity of *N*-methylamino acids has been shown to be invalid, the error having been due to misassignment of the peaks of the amino acid analysis. It transpires that under the conditions described, the L-alanine *N*-carboxyanhydride not only reacts poorly with the *N*-methylleucine but also gives a dipeptide with an unusually low ninhydrin color yield (4.6% of that for alanine, 6.4%

of that for Ala-Leu) making it very difficult to identify the Ala-MeLeu peak among the by-product peaks. A direct test for racemization was possible, however, for the isoleucines. Analysis (after hydrogenation) of the crude products of methylation indicated that Z-MeIle and Z-D-MeaIle contained, respectively, 1.1 and 0.0% of the diastereomer. In an indirect racemization test, *N*-methylleucine from the room-temperature methylation was converted to the benzyl ester *p*-toluenesulfonate (14) and the crude ester was coupled to Z-Ala by the mixed-anhydride method. The neutral product of the coupling was hydrogenated and the resulting dipeptide shown to contain 0.6% of the L,D-diastereomer.[4] Using the same method, it was shown that *N*-methylleucine, prepared by the saponification and subsequent hydrogenation of Z-MeLeu-OMe (1*b*) contained 9.6% of the D-isomer. *N*-Methylthreonine obtained from the crude *t*-butyl derivative had, before recrystallization, an optical rotation close to that reported in the literature. Also, crude MeThr(Bu^t) was homogeneous on t.l.c. and amino-acid analysis, indicating that little racemization had occurred.

It might be expected that amino acids with alkyl side-chains would be less prone to racemize during methylation than amino acids with electron-withdrawing side-chains. The optical integrity of products obtained by the methylation of amino acids of the latter type remains to be established by an unambiguous method.

In view of the racemization occurring during the saponification of *N*-benzyloxycarbonyl,*N*-methylamino-acid esters (6), this method of room temperature methylation should have wide applicability for the preparation of *N*-benzyloxycarbonyl,*N*-methylamino-acid derivatives for peptide synthesis.

Experimental

Materials and Methods

Sodium hydride was obtained from Fluka AG, Buchs, Switzerland, and J. T. Baker Chemical Company, Phillipsburg, New Jersey as 55 and 57% dispersions in oil respectively. Analysis showed the presence of 2–3% by weight of NaOH (15). Methyl iodide and tetrahydrofuran (THF) were products from Fisher Scientific Company, Pittsburgh, Pennsylvania. The THF was purified by distillation from KOH and then from sodium hydride. Amino acids were obtained from General Biochemicals, Chagrin Falls, Ohio, except for D-*allo*-isoleucine which was from ICN Nutritional Biochemicals, Cleveland, Ohio. Benzyloxycarbonylamino acids were prepared by the original procedure as described by Greenstein and Winitz (16). Z-Asp(OBu^t) and Z-Thr(Bu^t) were obtained as their dicyclohexylamine salts and were converted to the free acid using aqueous citric acid. Z-Glu(OBu^t) was obtained from Schwarz-Mann, Orangeburg, New York. Z-Asp(OBu^t)-OBu^t and Z-Glu(OBu^t)-OBu^t were prepared by the isobutylene method of Anderson and Callahan (17). Z-Asp(OMe)-OMe was the neutral product isolated from the reaction of Z-Asp with diazomethane. Hydrogenations were done in the presence of 10% palladium-on-charcoal catalyst at atmospheric pressure. Light petroleum refers to the 30–60° fraction, from Fisher Scientific Co.

Optical rotations were measured with a Perkin–Elmer model 141 polarimeter using a 1-dm tube. Melting points were taken by the capillary method, and are uncorrected. Elemental analyses were done by Organic Microanalyses, Montreal, on the compounds dried under *vacuo* over P_2O_5. Amino-acid analyses were carried out with a Beckman model 120B amino-acid analyzer with the eluting buffer at half flow rate to allow detection of the *N*-methylamino acids (13).

Proton n.m.r. (Varian T-60) and i.r. (Unicam SP 200) analyses were used routinely to check the product of methylation, using the *N*-methyl singlet at τ 7.0 and the >*N*—H stretch and amide II bands at 3280 and 1520 cm^{-1}, respectively. All products had spectra which were consistent with the assigned structures.

General Methylation Procedure

N-Benzyloxycarbonylamino acid or *t*-butyl derivative (10 mmol) and methyl iodide (5 ml; 80 mmol) were dissolved in THF (30 ml) and the solution was cooled to 0 °C in a flask protected from moisture. Sodium hydride dispersion (1.32 g; 30 mmol) was added cautiously with gentle stirring. The suspension was stirred at room temperature for 24 h. Ethyl acetate (50 ml) was then added (to consume the sodium hydride formed from the excess sodium hydride), followed by water, dropwise, to destroy the excess sodium hydride. The solution was evaporated to dryness, and the oily residue partitioned between ether (30 ml) and water (100 ml). The ether layer was washed with aqueous $NaHCO_3$ (50 ml), and the combined aqueous extracts acidified to p*H* 2 with 5 *N* HCl (for *t*-butyl derivatives, aqueous citric acid was used to p*H* 3). The product was extracted into ethyl acetate (2 × 50 ml), the extract was washed with water (2 × 50 ml), 5% aqueous sodium thiosulfate (2 × 50 ml; to remove iodine), and water (50 ml), dried over $MgSO_4$ and evaporated to give a pale yellow oil.

Z-MeAsp and Z-MeGlu

Z-MeAsp(OBu^t) and Z-MeGlu(OBu^t) were obtained as oils from the corresponding *t*-butyl derivative by the general procedure. The oils were dissolved in ether – light petroleum (1:1), dicyclohexylamine (1 ml) was added, the solutions were left overnight at 0 °C, and the precipitates were filtered off. The filtrates were washed with 5% aqueous citric acid (2×) and water (4×), dried ($MgSO_4$), and evaporated. The purified Z-MeAsp(OBu^t) (86%) and Z-MeGlu(OBu^t) (70%) were left in trifluoroacetic acid (5 ml/g) for 1 h and the solutions were evaporated.

[4]The synthesis of reference compounds and the chromatographic data for the analysis of the various diastereomers will be described in a subsequent paper (6*b*).

MeThr(Bu^t)

Z-MeThr(Bu^t) (0.79 g; 90%) was obtained from Z-Thr(Bu^t) as described for the other *t*-butyl derivatives, including purification through the dicyclohexylamine salt, and was hydrogenated in 65% aqueous ethanol (15 ml) for 4 h. Evaporation of the solution yielded a crystalline product (100% yield) which gave a single peak on amino-acid analysis (long column, p*H* 3.28 buffer). A sample crystallized from water–acetone sublimed at 269–271°, had $[\alpha]_D^{27}$ −53.1° (*c*, 1 in H_2O), and gave only one spot on analysis by t.l.c. The MeThr(Bu^t) was converted to MeThr using trifluoroacetic acid at room temperature for 2 h. The solution was evaporated, the residue dissolved in 1 *N* HCl, and the solution evaporated again. The residue was dissolved in water (5 ml), the solution was stirred with Dowex 50 (H^+ form) resin, and the resin was filtered off and washed with water until the filtrate contained no chloride ions. The product was eluted from the resin with 10 *N* NH_4OH (100 ml), the solution evaporated and the crystalline residue dried over P_2O_5. The product, m.p. 228–236°, $[\alpha]_D^{26}$ −32.0° (*c*, 1 in H_2O), had m.p. 238–240° after recrystallization from methanol, lit. m.p. 244–246°, $[\alpha]_D^{20}$ −35.9° (*c*, 2.3 in H_2O) (18).

Methylation of Z-Asp(OBu^t)-OBu^t, Z-Glu(OBu^t)-OBu^t and Z-Asp(OMe)-OMe

Methylations were carried out by the usual procedure, using 1.5 mol of sodium hydride and 4 mol of methyl iodide. The neutral product was isolated in ether from the ether–water partition, the ether solution was dried ($MgSO_4$) and evaporated to give an oil. N.m.r. and i.r. analyses indicated that complete *N*-methylation had occurred in each case. Deprotection of the *t*-butyl derivatives with trifluoroacetic acid for 2 h gave Z-MeAsp (64% yield, recrystallized) with $[\alpha]_D^{27}$ −21.6° (*c*, 1 in EtOH) and Z-MeGlu (61% yield, recrystallized) with $[\alpha]_D^{27}$ −13.8° (*c*, 1 in EtOH). Saponification of Z-MeAsp-(OMe)-OMe (1.0 g) with 2 *N* NaOH (10 ml) in methanol (10 ml) for 2 h gave Z-MeAsp (0.43 g) with $[\alpha]_D^{26}$ −5.4° (*c*, 1 in EtOH).

Attempted Methylation of Z-Asp, Z-Glu, and Z-Thr

After methylation using 4.5 mol of sodium hydride and 12 mol of methyl iodide, n.m.r. and i.r. analyses of the acidic products indicated that no *N*-methylation of Z-Asp and Z-Glu had taken place. For Z-Thr, n.m.r. analysis showed the presence of *N*-methyl (τ 7.0) and *O*-methyl (τ 6.7) groups with integral values corresponding to 60% *N*-methyl and 25% *O*-methyl. I.r. analysis showed that some >*N*—H was still present.

Methylation of Z-Met

(*a*) When the general procedure was used, the product was lost during the isolation due to its solubility in water.

(*b*) Z-Met (1.34 g; 5 mmol), methyl iodide (0.31 ml; 5 mmol), and sodium hydride dispersion (0.60 g; 15 mmol) were stirred in THF for 18 h. The acidic product was isolated by the general procedure as a pale yellow oil (1.05 g; 71%). N.m.r. and i.r. analyses indicated virtually complete methylation. Analysis of a weighed sample, after deprotection with 2 *N* HBr in acetic acid for 2 h, showed that the sample contained about 3.4% of Z-Met.

Methylation of Z-Trp

N.m.r. analysis of the oily product showed singlets at τ 6.3 and 7.1 in a 1:1 ratio.

Methylation of Z-Ala-Leu

Z-Ala-Leu (19) was methylated by the general procedure using 4.5 mol of sodium hydride and 12 mol of methyl iodide. The acidic product (yield 58% by weight) was an oil whose n.m.r. spectrum showed a singlet at τ 7.0 and no peaks at 2.7 and 4.9 for the benzyl protons. The equivalent weight determined by titration was 294. Required for the hydantoin **1**, 274.

Methylation of Z-Ala-MeLeu

Methylation of Z-Ala-MeLeu[4] by the general procedure gave an oil, 100% yield, with $[\alpha]_D^{19}$ −67.2° (*c*, 2 in EtOH), whose i.r. spectrum showed that no >*N*—H remained.

Racemization Tests

Z-MeLeu was hydrogenated in 80% acetic acid for 4 h and the solution was evaporated giving a white crystalline solid. This compound (0.47 g) was dissolved in benzyl alcohol (20 ml) and benzene (10 ml) containing *p*-toluenesulfonic acid (0.63 g), and the mixture was heated under reflux, the water formed being trapped in a Dean-Stark tube. The benzene was removed by evaporation, the product precipitated with a large volume of ether, and collected by filtration (0.82 g). This MeLeu-OBzl·TosOH (0.106 g) was dissolved in ethyl acetate (1 ml) containing triethylamine (0.035 ml) and the solution was added to the mixed anhydride formed from Z-Ala (0.056 g) and ethyl chloroformate (0.024 ml) in THF (1 ml) at 0 °C by the addition of triethylamine (0.035 ml). The mixture was stirred at 0° for 1 h and at room temperature for 1 h. After removal of the solvent, the total neutral fraction was extracted into ethyl acetate, the solution was dried and evaporated, and the residual oil was hydrogenated in acetic acid containing 2 *N* HCl (0.2 ml) for 8 h to give the free dipeptide. Analysis[4] showed the product to contain 0.6% of the L,D-isomer. A similar experiment using Z-MeLeu prepared by saponification of Z-MeLeu-OMe gave a product containing 9.6% of the L,D-isomer.

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