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AMINO ACIDS AND PEPTIDES. XLII. SYNTHESIS OF AN OCTAPEPTIDE SEQUENCE (A_{20}-A_{27}) OF RUBREDOXIN

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A protected octapeptide corresponding to a section found in the first half of the protein chain of rubredoxin has been prepared by standard peptide methods. Alternative approaches to this fragment are discussed in some detail. This work completes the series of subunits necessary to synthesize the molecule.

The electron-transfer protein rubredoxin offers an interesting preparation challenge, based in part on the chelated nature of the cystine residues, and on the relationship of the protein (1) to ferredoxin, another iron-containing enzyme, which is implicated in the fixation of nitrogen. In previous years there has been described a proposed synthetic approach to rubredoxin involving the formation of various protected fragments. These sections include A_1-A_{10} (2), A_{11}-A_{19} (3), A_{28}-A_{37} (4), A_{38}-A_{42} (5), and A_{43}-A_{53} (6). We now report the preparation of the remaining unit in this series, residues A_{20}-A_{27}, prolyl-aspartyl-threonyl-prolyl-aspartyl-glutaminyl-aspartyl-glycine.

The desired peptide contains two prolyl-aspartyl residues, so in order to minimize the effort involved, the synthesis was designed to utilize a common intermediate at these two points. To begin, N^{α}-benzyloxycarbonylglycine (I) was coupled to phenol in the presence of *N,N'*-dicyclohexylcarbodiimide to give phenyl N^{α}-benzyloxycarbonylglycinate (II) (7). Stirring compound II with hydrogen bromide-acetic acid then generated phenyl glycinate hydrobromide (III) (8). Hydrogenolysis of methyl N^{α}-benzyloxycarbonyl-*β-t*-butyl-L-aspartate (IV) formed methyl *β-t*-butyl-L-aspartate (V), which was joined to N^{α}-benzyloxycarbonyl-L-glutamine (VI) by *N,N'*-dicyclohexylcarbodiimide to afford methyl N^{α}-benzyloxycarbonyl-L-glutaminyl-*β-t*-butyl-L-aspartate (VII). Hydrolysis of dipeptide VIII with aqueous sodium hydroxide yielded the corresponding acid (VIII). Addition of *N,N'*-dicyclohexylcarbodiimide to a mixture of acid VIII and amine III in the presence of triethylamine furnished phenyl N^{α}-benzyloxycarbonyl-L-glutaminyl-*β-t*-butyl-L-aspartylglycinate (IX).

It is to be noted that the carboxyl terminus of tripeptide IX was blocked as a phenyl ester (9). Such derivatives are readily cleaved by treatment with peroxide anion at pH 10.5. To verify this point, tripeptide IX was reacted with basic hydrogen peroxide solution to yield N^{α}-benzyloxycarbonyl-L-glutaminyl-*β-t*-butyl-L-aspartylglycine (X). This favorable result then permitted a return to the main synthesis route. Subsequently, hydrogenolysis of compound IX was carried out in the presence of one equivalent of *p*-toluenesulfonic acid in order to trap the liberated amine. Note that any free base here at this point might attack the reactive phenyl ester present in the reaction mixture. Work-up produced the crystalline salt, phenyl L-glutaminyl-*β-t*-butyl-L-aspartylglycinate *p*-toluenesulfonate (XI).

An active ester coupling between *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-prolinate (XII) and amine V supplied methyl N^{α}-benzyloxycarbonyl-L-prolyl-*β-t*-butyl-L-aspartate (XIII). Removal of the N^{α}-benzyloxycarbonyl group in the usual fashion provided the amine (XIV), which on

combination with N^{α}-benzyloxycarbonyl-O-*t*-butyl-L-threonine in the presence of *N*, *N'*-dicyclohexylcarbodiimide led to methyl N^{α}-benzyloxycarbonyl-O-*t*-butyl-L-threonyl-L-prolyl-*β*-*t*-butyl-L-aspartate (XVI). Hydrolysis of the methyl ester XVI with cold, dilute alkali converted it to the acid (XVII); the progress of the reaction was easily followed by nuclear magnetic resonance (n.m.r.) spectroscopy. The acid XVII was combined with the amine XI so as to obtain phenyl N^{α}-benzyloxycarbonyl-O-*t*-butyl-L-threonyl-L-prolyl-*β*-*t*-butyl-L-aspartyl-L-glutaminyl-*β*-*t*-butyl-L-aspartylglycinate (XVIII). Cleavage of the benzyloxycarbonyl moiety with 10% palladium-on-charcoal formed the hexapeptide amine (XIX). Dipeptide XIII was hydrolyzed under the same conditions as described for tripeptide XVI to give N^{α}-benzyloxycarbonyl-L-prolyl-*β*-*t*-butyl-L-aspartic acid (XX). A final mixed anhydride step between acid XX and amine XIX yielded the desired octapeptide, phenyl N^{α}-benzyloxycarbonyl-L-prolyl-*β*-*t*-butyl-L-aspartyl-O-*t*-butyl-L-threonyl-L-prolyl-*β*-*t*-aspartyl-L-glutaminyl-*β*-*t*-butyl-L-aspartylglycinate (XXI). The amino acid analysis was also satisfactory and established that the desired compound was now available. The overall route is shown in Fig. 1 and the physical properties of the intermediates are summarized in Table 1.

An alternative original stepwise preparative scheme encountered several difficulties, which will be described now. Methyl glycinate (XXII) was coupled to N^{α}-benzyloxycarbonyl-*β*-*t*-butyl-L-aspartic acid (XXIII) by a mixed anhydride to afford methyl N^{α}-benzyloxycarbonyl-*β*-*t*-butyl-L-aspartylglycinate (XXIV). Hydrogenolysis in methanol provided the corresponding amine (XXV), which, unfortunately, quickly cyclized to the diketopiperazine derivative (XXVI). Repetition of the reaction in a methanol-acetic acid medium

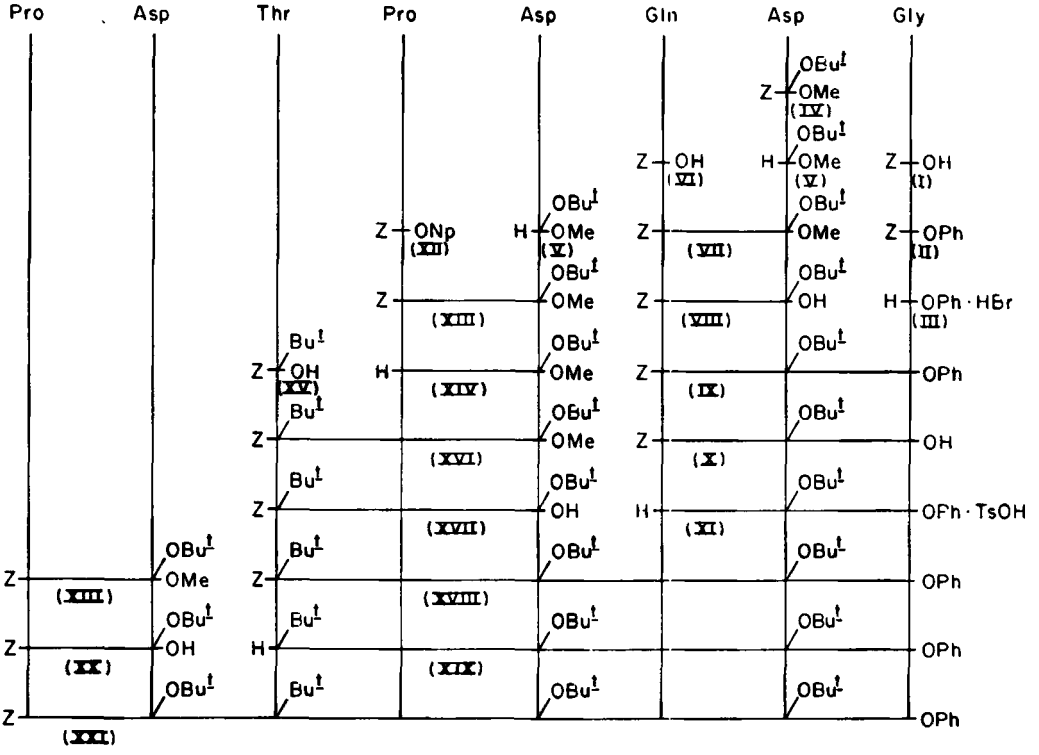

FIGURE 1

Schematic diagram of the synthesis of the protected A_{20}-A_{27} octapeptide (XXI) of rubredoxin: Z, benzyloxycarbonyl; Bu^t, *t*-butyl ether; OBu^t, *t*-butyl ester; OMe, methyl ester; ONp, *p*-nitrophenyl ester; OPh, phenyl ester; HBr, hydrobromide; TsOH, *p*-toluenesulfonate.

TABLE 1

Intermediates prepared for the synthesis of the A_{20}-A_{27} sequence of rubredoxin

Number	Compound[a]	Formula	Molecular weight	Method[b]	Reaction solvent[c]	Yield %	Crystallization solvent[a]	Melting point	R_f[e]	Optical rotation[f]	Microanalysis Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
II	Z-Gly-OPh	$C_{16}H_{15}NO_4$	285.29	DCCI	DCM	75	EtOAc-Pet. Ether	66–67	–	–	–	–	–	–	–	–
III	H-Gly-OPh · HBr	$C_8H_{10}BrNO_2$	232.01	HBr · HOAc	HOAc	78	MeOH-Ether	214–216	–	–	–	–	–	–	–	–
VII	Z-Gln-Asp(OBu^t)-OMe	$C_{22}H_{31}N_3O_8$	465.49	DCCI	THF	80	EtOH	155–156	–	–	–	–	–	–	–	–
VIII	Z-Gln-Asp(OBu^t)-OH	$C_{21}H_{29}N_3O_8$	451.56	NaOH	MeOH	75	EtOH	158–160	–	–	–	–	–	–	–	–
IX	Z-Gln-Asp(OBu^t)-Gly-OPh	$C_{29}H_{36}N_4O_{10}$	600.63	DCCI	DMF	81	MeOH	198–199	0.55(B)	−22.8	57.99	6.04	9.33	57.85	6.00	9.21
X	Z-Gln-Asp(OBu^t)-Gly-OH	$C_{23}H_{32}N_4O_{10}$	524.52	H_2O_2	DMF	–	–	–	–	–	–	–	–	–	–	–
XI	H-Gln-Asp(OBu^t)-Gly-OPh · TsOH	$C_{28}H_{38}N_4O_{10}S$	622.68	H_2	DMF	–	–	–	0.05(B)	–	–	–	–	–	–	–
XIII	Z-Pro-Asp(OBu^t)-OMe	$C_{21}H_{32}N_2O_6$	408.48	*p*-Np	THF	75	–	Oil	0.75(A)	−26.0	–	–	–	–	–	–
XIV	H-Pro-Asp(OBu^t)-OMe	$C_{13}H_{26}N_2O_4$	274.35	H_2	MeOH	–	–	Oil	0.10(A)	–	–	–	–	–	–	–
XVI	Z-Thr(Bu^t)-Pro-Asp(OBu^t)-OMe	$C_{29}H_{47}N_3O_8$	565.69	DCCI	DCM	62	–	Oil	0.68(A)	−18.0	–	–	–	–	–	–
XVII	Z-Thr(Bu^t)-Pro-Asp(OBu^t)-OH	$C_{28}H_{43}N_3O_8$	549.65	NaOH	MeOH	65	Ether-Pet. Ether	76–77	0.10(B)	−21.3	61.18	7.89	7.65	60.92	7.81	7.38
XVIII	Z-Thr(Bu^t)-Pro-Asp(OBu^t)-Gln-Asp(OBu^t)-Gly-OPh	$C_{50}H_{71}N_7O_{16}$	1026.16	MA	DMF	79	EtOH	89–90	0.40(B)	−28.0	58.53	6.97	9.55	58.69	7.10	9.85
XIX	H-Thr(Bu^t)-Pro-Asp(OBu^t)-Gln-Asp(OBu^t)-Gly-OPh · TsOH	$C_{49}H_{73}N_7O_{17}S$	1064.19	H_2	DMF	–	–	–	0.05(B)	–	–	–	–	–	–	–
XX	Z-Pro-Asp(OBu^t)-OH	$C_{20}H_{28}N_2O_6$	392.45	NaOH	MeOH	78	Diisopropyl Ether	130–132	–	−21.8	61.21	7.19	7.13	61.52	7.40	7.01
XXI	Z-Pro-Asp(OBu^t)-Thr(Bu^t)-Pro-Asp(OBu^t)-Gln-Asp(OBu^t)-Gly-OPh	$C_{63}H_{91}N_9O_{20}$	1294.43	H_2	DMF	74	MeOH-EtOAc	88–91	0.35(B)	−31.0	–	–	–	–	–	–
XXIV	Z-Asp(OBu^t)-Gly-OMe	$C_{19}H_{26}N_2O_7$	394.42	MA	THF	75	–	Oil	0.90(B)	–	–	–	–	–	–	–
XXV	H-Asp(OBu^t)-Gly-OMe	$C_{11}H_{20}N_2O_5$	260.29	H_2	HOAc	90	–	Oil	0.20(B)	–	–	–	–	–	–	–
XXVI	Asp(OBu^t)-Gly	$C_{10}H_{16}N_2O_4$	228.25	H_2	MeOH	100	MeOH	213–215	–	–	52.62	7.07	12.27	52.72	6.76	12.05
XXVIII	Z-Gln-Asp(OBu^t)-Gly-OMe	$C_{24}H_{34}N_4O_9$	522.63	MA	THF	62	MeOH-H_2O	164–167	0.40(B)	−19.4	55.16	6.56	10.72	55.28	6.67	10.84
XXIX	H-Gln-Asp(OBu^t)-Gly-OMe	$C_{16}H_{28}N_4O_7$	388.42	H_2	MeOH	90	–	Glass	–	–	–	–	–	–	–	–
XXXI	Z-Asp(OBu^t)-Gln-Asp(OBu^t)-Gly-OMe	$C_{32}H_{47}N_5O_{12}$	693.77	*p*-Np	M	82	MeOH-H_2O	185–189	–	−20.8	55.38	6.79	10.10	55.39	6.86	10.03
XXXII	Z-Gly-OCH_2CCl_3	$C_{12}H_{12}Cl_3NO_4$	340.60	DCCI	THF	70	–	Oil	0.90(A)	–	–	–	–	–	–	–
XXXIII	H-Gly-OCH_2Cl_3 · HBr	$C_4H_7BrCl_3NO_2$	287.66	HBr · HOAc	HOAc	90	–	Deliquescent solid	–	–	–	–	–	–	–	–
XXXIV	Z-Asp(OBu^t)-Gly-OCH_2CCl_3	$C_{20}H_{24}Cl_3N_2O_7$	510.78	MA	THF	85	–	Oil	–	–	–	–	–	–	–	–
XXXV	Z-Gly-NHNHOCOCH_2CCl_3	$C_{13}H_{13}Cl_3N_3O_5$	397.63	DCCI	THF	90	EtOAc-Ether	119–120	0.80(B)	–	–	–	–	–	–	–
XXXVI	H-Gly-NHNHOCOCH_2CCl_3	$C_5H_8BrCl_3N_3O_3$	344.43	HBr · HOAc	HOAc	85	–	Deliquescent solid	–	–	–	–	–	–	–	–
XXXVII	Z-Asp(OBu^t)-Gly-NHNHOTr	$C_{21}H_{26}Cl_3N_4O_8$	568.82	MA	THF	–	–	Oil	0.85(B)	–	–	–	–	–	–	–

[a] All amino acids with the exception of glycine are of the "L" configuration.

[b] DCCI = *N*,*N*′-dicyclohexylcarbodiimide; H_2 = hydrogenation in the presence of 10% palladium-charcoal; HBr-HOAc, 30% hydrogen bromide in acetic acid; H_2O_2 = 30% hydrogen peroxide; MA = *N*-methylmorpholine and isobutyl chloroformate; NaOH = 1 *N* sodium hydroxide; *p*-Np = *p*-nitrophenyl ester.

[c, d] DCM = dichloromethane; DMF = *N*,*N*′-dimethylformamide; EtOAc = ethyl acetate; EtOH = ethanol; Ether = diethyl ether; H_2O = water; HOAc = acetic acid; MeOH = methanol; Pet. Ether = petroleum ether (30–60°); THF = tetrahydrofuran.

[e] Thin-layer chromatographic values were obtained with silica gel GF-254 as the support and iodine or ninhydrin as the detection agents. The eluent employed was chloroform-methanol: A(97:3); B(90:10).

[f] Optical rotations were measured at 20°, *c* = 1. *N*,*N*′-Dimethylformamide was used for IX, XVIII, XXI, XXVIII, and XXXI, while XIII, XVI, XVII, and XX were dissolved in chloroform.

generated the stable amine salt and this material was combined with the protected glutamic acid derivatives XXVIII by a pivalic acid mixed anhydride reaction to supply methyl N^{α}-benzyloxycarbonyl-L-glutaminyl-β-*t*-butyl-L-aspartylglycinate (XXVIII). This particular method is known to minimize the possibility of intramolecular attack of the glutamine's activated acyl function by the amide nitrogen and would thus avoid another undesired cyclization. Hydrogenation as usual produced the amine (XXIX) and prompt treatment with *p*-nitrophenyl N^{α}-benzyloxycarbonyl-β-*t*-butyl-L-aspartate (XXX) furnished methyl N^{α}-benzyloxycarbonyl-β-*t*-butyl-L-aspartyl-L-glutaminyl-β-*t*-butyl-L-aspartylglycinate (XXXI).

Before continuing with the synthesis, it was necessary to ascertain the stability of the *t*-butyl groups on the aspartic acid residues with respect to either saponication or hydrazinolysis conditions. In order to test this idea, the tetrapeptide XXXI was saponified at room temperature for 45 min with 1 equivalent of base and the resultant free acid isolated and subjected to n.m.r. analysis. Integration of the signals showed about 50% of the expected *t*-butyl signal as compared to the benzyloxycarbonyl signal. A similar result was obtained in a variety of different polar solvents including methanol, dioxane, and acetone. It appeared, then, that basic hydrolysis of the glycyl methyl ester to give the corresponding glycyl acid was impossible without simultaneous disruption of the aspartyl *t*-butyl esters.

Next, hydrazinolysis was attempted by treating compound XXXI with hydrazine hydrate in methanol. The product of this reaction also gave a n.m.r. spectrum with a deficient *t*-butyl signal. Presumably, one of the *t*-butyl esters was attacked by hydrazine to form a sidechain hydrazine (10), which completely ruined the usefulness of this specific tetrapeptide.

At this point it seemed clear that some other means of blocking the C-terminal glycine would be necessary here. One possibility might be a trichloroethyl ester, which is known to be removed by stirring with zinc dust and 90% acetic acid (11). To test the feasibility of this idea, 2, 2, 2-trichloroethyl N^{α}-benzyloxycarbonylglycinate (XXXII) was converted into the deblocked hydrobromide salt (XXXIII) by addition of hydrogen bromide-acetic acid. A mixed anhydride condensation with the blocked aspartic acid XXIII then gave 2, 2, 2-trichloroethyl N^{α}-benzyloxycarbonyl-β-*t*-butyl-L-aspartylglycinate (XXXIV). Unfortunately, zinc and 90% acetic acid failed to deblock dipeptide XXXIV. The use of more severe conditions, such as amalgamated zinc or formic acid, instead of acetic acid, resulted in loss of the β-*t*-butyl ester, as well as removal of the 2, 2, 2-

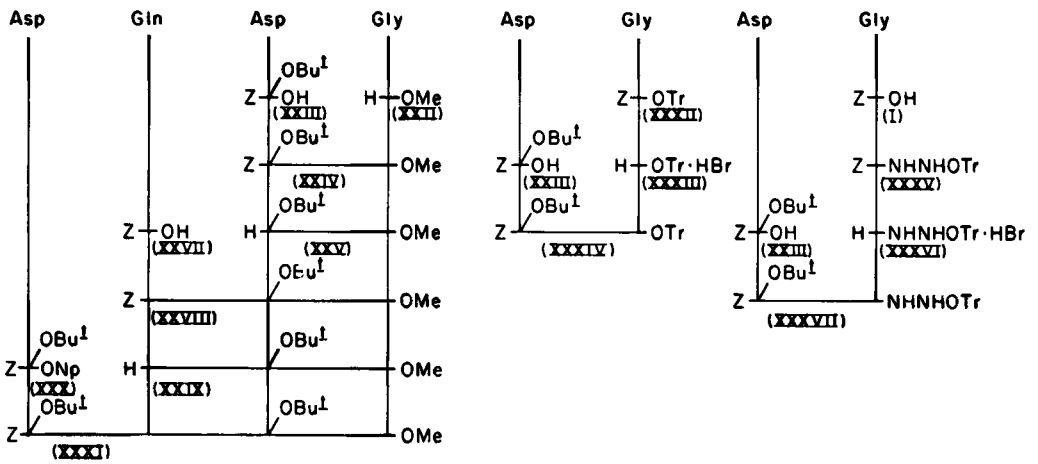

FIGURE 2

Schematic diagram of the alternative approach to the synthesis of the protected A_{20}-A_{27} octapeptide (XXI) of rubredoxin: Z, benzyloxycarbonyl; OBu^t, *t*-butyl ester; OMe, methyl ester; ONp, *p*-nitrophenyl ester; OTr, 2, 2, 2-trichloroethyl ester; ONHNHOTr, 2, 2, 2-trichloroethoxycarbonylhydrazide; HBr, hydrobromide.

trichloroethyl group. It appeared that this type of protection would be of no help to the problem at hand.

Another alternative was investigated at this time. The 2, 2, 2-trichloroethoxycarbonyl group has been used for the masking of urethanes (12). In order to test the utility of this blocking agent, N^{α}-benzyloxycarbonylglycine (I) was joined to 2, 2, 2-trichloroethoxycarbonylhydrazine with the aid of *N*, *N'*-dicyclohexylcarbodiimide to yield N^{α}-benzyloxycarbonyl-glycine 2, 2, 2-trichloroethoxycarbonylhydrazide (XXXV). Treatment of the hydrazide XXXV with hydrogen bromide provided glycyl 2, 2, 2-trichloroethoxycarbonylhydrazide hydrobromide (XXXVI), which was combined with the aspartic acid intermediate XXIII to obtain N^{α}-benzyloxycarbonyl-L-aspartylglycine 2, 2, 2-trichloroethoxycarbonylhydrazide (XXXVII). Zinc dust in acetic acid readily removed the hydrazide blocking group in XXXVII, and, most importantly, the nuclear magnetic spectrum showed no loss of the β-*t*-butyl ester signal. It appeared that a satisfactory protection scheme had been developed here. However, when dipeptide XXXVII was hydrogenated using standard conditions, severe decomposition took place. The odor of hydrogen chloride was quite noticeable, which was attributed to simultaneous hydrogenolysis of the 2, 2, 2-trichloroethoxycarbonylhydrazide. This result led to discontinuation of our interest in the 2, 2, 2-trichloroethyl group as a protecting group in this fragment.

One last method was evaluated now. Esters based on 4-(methylthiol) phenyl have been suggested for carboxyl protection, since peroxide treatment conveniently converts them into activated 4-(methylsulfonyl) phenyl esters (13). To investigate this idea, 4-(methylthio) phenyl N^{α}-benzyloxycarbonylglycine (XXXVIII) was made by means of a *N*, *N'*-dicyclohexylcarbodiimide coupling between intermediate I and 4-(methylthio) phenol. Attempts to remove the benzyloxycarbonyl group of XXXVIII were futile, although excess palladium-on-charcoal, sulfided catalyst, or the addition of boron trifluoride-etherate were tried in various combinations. Since sulfur, in its lower oxidation states, is well known as a potent catalyst poison, this result was not unexpected. In view of these various limitations, our interest terminated in this group, too.

It is believed that the above observations made by us will be of general interest to workers in the field, especially with respect to the use of some new protecting groups that have been suggested recently.

Finally, we wish to call attention to the technique of monitoring the various conversions discussed here with nuclear magnetic spectroscopy. It is quite easy to verify the actual existence and number of N^{α}-benzyloxycarbonyl groups (δ 7.3 and 5.2), *t*-butyl esters (δ 1.2), *t*-butyl ethers (δ 1.5), and methyl esters (δ 3.8) in deuteriochloroform, as they give rise to simple, singlet patterns. Note that the amino hydrogen peak is very solvent dependent, so some care should be used in the assignment of this absorption signal. With such data, we can frequently follow a reaction in a sample tube or characterize an oily product in a facile fashion. This information is a valuable adjunct to the usual analytical procedures and should be used as a general tool by peptide chemists.

EXPERIMENTAL PROCEDURES

All conversions involved known procedures and followed the usual reaction conditions. Work-up conditions generally used reduced pressure to remove the solvent and a combination of washes involving dilute, aqueous solutions of citric acid and sodium bicarbonate.

Melting points were determined on a Reichart "Thermopan" unit and are uncorrected. Evaporations were performed under reduced pressure (water pump) with a rotatory apparatus at minimum temperature, while high-boiling solvents were removed at vacuum pressure (0.2–0.5 mm). Magnesium sulfate was used for drying purposes. *N,N'*-Dimethylformamide was spectroquality; other solvents were reagent grade. Microanalyses were furnished by Galbraith Laboratories, Knoxville, Tennessee and the amino acid analysis was done by AAA Laboratories, Seattle, Washington.

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