TABLE 1. Results of the Potentiometric Titration of Mixtures of BOC-AAs and AAs (n = 6, t = 2.57, P = 0.95)

Composition of the mixture	Differential titration			Combined methods		
	$\bar{C}$, r	$S_r \cdot 10^2$	$\pm\sigma \cdot 10^2$	$\bar{C}$, r	$S_r \cdot 10^2$	$\pm\sigma \cdot 10^2$
β-Phenyl-α-alanine + BOC-β-phenyl-α-alanine	0,0404	5,53	14,22	0,0619	5,05	12,99
	0,1034	3,46	8,89	0,1004	3,56	9,15
Proline + BOC-proline	0,0401	4,98	12,82	0,0654	4,07	12,77
	0,1004	3,12	8,02	0,0968	4,00	10,28
Serine + BOC-serine	0,0501	4,55	11,60	0,0689	5,05	13,02
	0,1031	3,47	8,92	0,1054	4,16	10,68
Valine + BOC-valine	0,0502	4,79	12,33	0,0593	6,03	15,50
	0,1038	3,88	9,97	0,1027	4,46	11,46
Tryptophan + BOC-tryptophan	0,0680	3,83	9,85	0,0818	4,64	11,92
	0,1123	3,98	10,23	0,1418	2,78	7,16
Leucine + BOC-leucine	0,0512	5,59	14,37	0,0763	4,78	12,14
	0,1073	3,00	12,68	0,1370	3,16	8,13

2. The methods can be used for the analysis of industrial samples of BOC-AAs.

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SYNTHESIS OF DINITROPHENYLTETRAPEPTIDES AS CHROMOPHORIC SUBSTRATES OF ENDOPROTEINASES

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The synthesis has been performed of ten tetrapeptides of the general formula Dnp-Gly-Gly-X-Arg-OH, where X = Val, Phe, Abu, Asp(OBu^t), Asp, Met, D-Phe, Ser, Thr, or Trp. The synthesis was carried out with Dnp-Gly-Gly-ONp, activated esters of protected amino acids, and arginine with an unprotected carboxy group. The coefficients of molar extinction of the tetrapeptides at 660 nm are given. It has been shown that the peptides can be used to determine the activities of neutral and alkaline proteinases from various sources, the peptides with X = Phe, Met, and Abu exhibiting the highest sensitivity to enzymatic hydrolysis.

The activities of proteolytic enzymes are determined both with the use of protein substrates and in relation to low-molecular-weight peptide substrates of definite structure. In the latter case, the possibility exists of differentiating proteinases with different specificities and also of achieving standardization of the conditions of the determination more easily. To increase the sensitivity of spectrometric analysis it is possible to introduce into the substrates a chromophoric grouping shifting the absorption spectrum of the peptide into the visible region; for example, an aromatic azo group [1] or a 2,4-dinitrophenyl resi-

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TABLE 1. Yields and Constants of the 2,4-Dinitrophenyltetrapeptides

Compound	Yield, %	mp, °C (solvent)	$[\alpha]_D^{20}$, degrees (concentration, solvent)	Rf in system		$\varepsilon_{360} \cdot 10^{-4}$
				1	2	
I. Dnp-Gly-Gly-Val-Arg-OH	89	172—174 (lyoph.)	—32 (*c* 1; DMFA)	0,54	0,29	1,49
II. Dnp-Gly-Gly-Phe-Arg-OH·CH_3OH	90	188—196 (methanol—ether	—33 (*c* 1; DMFA)	0,62	0,34	1,28
III. Dnp-Gly-Gly-Abu-Arg-OH	66	195—198 (H_2O)	—32 (*c* 1; DMFA)	0,54	0,29	1,67
IV. Dnp-Gly-Gly-Asp(OBu^t)-Arg-OH	57	190 decomp. (lyoph.)	—50 (*c* 1; DMFA)	0,62	0,34	1,17
V. Dnp-Gly-Gly-Asp-Arg-OH·CF_3COOH	66	210—220 (lyoph.)	—83 (*c* 1; DMFA)	0,40	0,20	1,88
VI. Dnp-Gly-Gly-Met-Arg-OH·H_2O	75	166—174 (H_2O)	—32 (*c* 1; DMFA)	0,50	0,28	*
VII. Dnp-Gly-Gly-*D*-Phe-Arg-OH·1,5H_2O	72	190—205 (lyoph.)	Not det.	0,60	0,34	1,51
VIII. Dnp-Gly-Gly-Ser-Arg-OH	68	170—175 (H_2O)	—68 (*c* 1; DMFA)	0,37	0,22	*
IX. Dnp-Gly-Gly-Thr-Arg-OH·H_2O	71	166—186 (H_2O)	—33 (*c* 1; DMFA)	0,36	0,30	1,78
X. Dnp-Gly-Gly-Trp-Arg-OH·2H_2O	53	218—222 (lyoph.)	—30 (*c* 1; DMFA)	0,19	0,08	0,68

*The peptide was insufficiently homogeneous according to the results of high-performance liquid chromatography.

due [2-4]. Thus, to determine collagenase and to detect a proteolytic activity of blood serum enzymes, such N^{α}-dinitrophenylated peptides* have been proposed as Dnp-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH and Dnp-Leu-Gly-Ile-Ala-Gly-Arg-OH [2]. For the testing of thermolysin and neutral protosubtilin, V. M. Stepanov et al. have developed simpler synthetic substrates — Dnp-Gly-Gly-Val-Arg-OH (I) [3] and a number of its analogs and, in particular, Dnp-Gly-Gly-Phe-Arg-OH (II) [4].

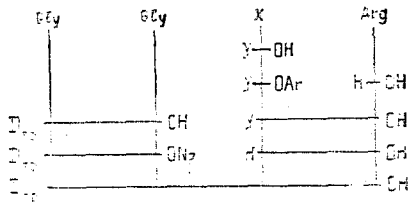
We have shown the sensitivity of peptides (I) and (II) and their analogs to hydrolysis by proteinases from various sources [5]. In the present paper we describe the synthesis and spectral characteristics of a number of 2,4-dinitrophenyltetrapeptides with C-terminal arginine and illustrate the possibility of their utilization. The choice of amino-acid residues in the Gly—X bond undergoing cleavage was determined primarily by the desirability of introducing hydrophobic amino acids, which have an affinity for the active centers of many enzymes. At the same time, in the determination of the N-terminal groups in a casein hydrolysate obtained under the action of an industrial preparation of alkaline protosubtilin, we found serine, threonine, and aspartic and glutamic acids; in view of this, the first three amino acids were also included in model peptides intended for the analysis of the specificity of alkaline protosubtilin.

The synthesis of the tetrapeptide (I) has been carried out previously starting with the methyl ester of arginine [3], and, in the last stage, performing the hydrolysis of the methyl ester by trypsin. The scheme that we propose for the synthesis is shorter by two stages, since it uses arginine with an unprotected carboxy group.

The dipeptides were obtained by the reaction of an activated ester of a protected amino acid with arginine in anhydrous dimethylformamide [6]. The completeness of the occurrence of the reaction was followed with the aid of TLC from the disappearance of the free arginine. After the precipitation with ether of the dipeptide formed, it was purified by recrystallization or reprecipitation. Dipeptides containing threonine and serine were obtained with the aid of pentachlorophenyl esters; after isolation from the reaction mixtures they were purified by preparative chromatography on plates coated with silica gel.

To deblock the dipeptides, depending on the protective groups used, we employed solutions of hydrogen bromide in glacial acetic acid, and solutions of hydrogen chloride in dioxane or trifluoroacetic acid. The tryptophan-containing peptide Z-Trp-Arg-OH was subjected to hydrogenolysis by boiling in ethanol in the presence of palladium black and cyclohexene [7]. The

*Here and below, if not specially mentioned, all the amino acids residues other than glycine have the L configuration.



Scheme of the synthesis of 2,4-dinitrophenyltetrapeptides: Y = Z- (benzyloxycarbonyl-); Boc- (tert-butoxycarbonyl-); Nps- (o-nitrophenylsulfenyl-); Np- (p-nitrophenyl-); Pcp- (pentachlorphenyl-); Tcp- (2,4,5-trichlorophenyl-), or Su- (N-succinimidyl-) (see Table 3).

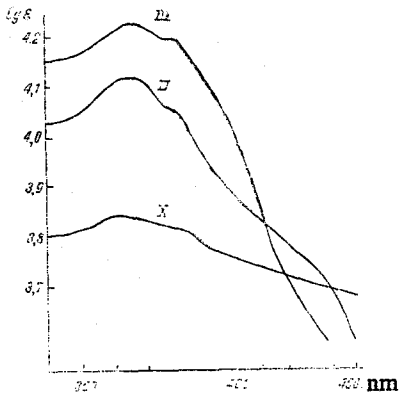


Fig. 1. UV spectra in the 340-450 nm region of the tetrapeptides (II), (III), and (X).

unprotected dipeptides obtained after deblocking in the form of salts were dissolved in water, and the solutions were treated with IRA-410 anion-exchange resin in the OH^- form and were then lyophilized. They were of satisfactory purity according to TLC and were used in the subsequent syntheses without additional purification.

In attempts to deblock the dipeptide Boc-Asp(OBu^t)-Arg-OH with a solution of hydrogen chloride in dioxane or with trifluoroacetic acid, a mixture of products was invariably obtained; in view of the unsatisfactory result of the simultaneous elimination of the protective groups, selective splitting out of the Boc protection with the aid of 85% formic acid [8] was performed. The β-tert-butyl ester of aspartylarginine so obtained was used in the synthesis of the tetrapeptides (IV) and (V).

All the tetrapeptides were obtained by the interaction of the deblocked dipeptides with the p-nitrophenyl ester of 2,4-dinitrophenylglycylglycine (prepared by the carbodiimide method, mp 209°C) in anhydrous dimethylformamide. After the precipitation of the peptides from the reaction mixture with a mixture of ethyl acetate and ether, their separation from the solution and their drying in vacuum, they were dissolved in water, and the solutions were washed free with ethyl acetate from an excess of activated ester, and were lyophilized. For analysis, the tetrapeptides were crystallized or reprecipitated from suitable solvents (Table 1). Their individualities were confirmed with the aid of TLC, high-performance liquid chromatography, and elementary analysis and, in individual cases, analysis of their amino-acid compositions.

The principle of the determination of enzymatic activities with the substrates obtained consisted in the fact that the colored hydrolysis product obtained after the necessary time of incubation of the proteinase with the substrate was extracted from the reaction mixture and determined spectrophotometrically [3]; the formation of one and the same product, Dnp-Gly-Gly-OH, from different tetrapeptides was confirmed with the aid of TLC. Its molar extinction coefficient at 360 nm was taken as 15,000 [3]. By analogy, the same value was taken for the tetrapeptides and was used for calculating their concentrations in the preparation of working solutions of them [3]. The molar extinction coefficient of a 2,4-dinitrophenyl(amino acid)

depends on the nature of the amino acid: thus, the corresponding ε_{360} values are 15,890 for Dnp-glycine, 17,400 for ε-Dnp-lysine and Dnp-ε-aminocaproic acid, and 18,300 for Dnp-(aspartic acid) [9]. It is also known that the intensity of the absorption of light by the Dnp-(amino acid)s at 360 nm is weakened as the result of complex formation with tryptophan [9].

The UV spectra of the tetrapeptides were recorded in the 300-500 nm region. In fact, they all have an absorption maximum at 360 nm (Fig. 1) but they differ with respect to the molar extinction coefficients (see Table 1). Consequently, one and the same optical density of the solutions corresponds to different molar concentrations of the tetrapeptides (I-X). Therefore, to obtain comparable results on a number of substrates it is desirable to prepare solutions of the latter by weight in the light of results of elementary analysis. With the peptide (III) as example, it was also shown that the optical density of its solution at 360 nm scarcely depends on the pH at pH 7-10; keeping the peptide in the light for a month did not affect its stability either in solution or in the solid state.

All the tetrapeptides obtained were tested as substrates for industrial preparations of neutral and alkaline protosubtilins and a commercial alkaline proteinase "subtilisin Carlsberg" (Sigma), and also for thrombin and blood plasma. It can be seen from the results given in Table 2 that substrates of the type under consideration may be selected not only for neutral but also for alkaline proteinases, which possess a narrower specificity. The advantage over the subtilisin substrates proposed previously — tripeptide p-nitroanilides [10, 11] — consists in their better solubility in water and, in particular, the possibility of working with unpurified enzyme preparations: Insoluble impurities and pigments do not interfere with the determination, since the measurement of optical density is carried out after extraction of the cleavage product to be determined, Dnp-Gly-Gly-OH.

EXPERIMENTAL

Chromatographic Methods. Thin-layer chromatography was carried out on plates with a fixed layer of silica gel Si-60 (Merck) in the following solvent systems: 1) butan-1-ol—acetic acid—water (4:1:1), and 2) butan-2-ol—3% ammonia (5:2). Preparative separation was carried out on plates coated with silica gel of type PSC (Merck) with a layer thickness of 2 mm in system 2. High-performance liquid chromatography was performed in a Spectrophysics SR-8000 instrument, with spectrophotometric detection at 360 nm in a methanol—0.1 M phosphate buffer, pH 4.7, gradient.

Analytical Methods. Amino-acid compositions were determined after hydrolysis with 6 N HCl at 110°C for 24 h on a Beckman Multichrom B amino-acid analyzer. Optical rotations were measured on a BPNL-3 instrument. UV spectra were recorded on a Specord UV-Vis automatic spectrometer. Molar extinction coefficients at 360 nm were measured on a SF-26 spectrophotometer.

The N-Protected Dipeptides (XI-XIX). A suspension of 1 mmole of arginine in 3 ml of dimethylformamide (DMFA) was treated with 1.2-1.5 mmole of an activated ester of an N-protected amino acid (Table 3). In the case of the pentachlorophenyl esters, an equivalent amount of triethylamine was added. The reaction mixture was stirred at room temperature for 24-72 h, with the completeness of the reaction being monitored with the aid of TLC from the disappearance of the arginine. Then the mixture was poured with stirring into 100 ml of ether, and the precipitate that deposited was filtered off and carefully washed with ether, dried in vacuum, and if necessary, subjected to additional purification (see Table 3).

TABLE 2. Relative Sensitivities of the 2,4-Dinitrophenyltetrapeptides to Cleavage by Various Enzymes

Enzyme preparation	Tetrapeptide substrate, Dnp-Gly-Gly-X-Arg-OH									
	X=Val I	Phe II	Abu III	Asp(OBu^t) IV	Asp IV	Met VI	D-Phe VII	Ser VIII	Thr IX	Trp X
Neutral protosubtilin	++	+	++	+	—	+	—	—	—	—
Alkaline protosubtilin	—	++	++	+	—	+	—	—	—	—
Subtilisin Carlsberg	—	+	+	—	—	—	—	—	—	—
Thrombin	—	+—	—	—	—	—	—	—	—	—
Blood plasma	—	++	++	+	+	++	—	+	+	—
Proteinase from the fungus Flammulina velutipes	+	++	—	—	—	+	—	—	—	—
Acid proteinase from Aspergilus oryzae	—	++	+	—	—	++	—	—	—	+

TABLE 3. Yields and Constants of the N-Protected Dipeptides

Compound	Activated ester used in the synthesis	Yield, %	mp, °C (solvent)	$[\alpha]_D^{20}$, degrees (concentration, solvent)	R_f in system	
					1	2
XI. Nps-Val-Arg-OH·0,5 C_2H_5OH	Nps-Val-ONp [12]	82	155—165 (C_2H_5OH)	—86 (*c* 1; DMFA)	0,75	0,41
XII. Boc-Phe-Arg-OH	Boc-Phe-OSu [13]	95	135—140 (methanol—ether)	—12 (*c* 1; DMFA)	0,66	0,16
XIII. Boc-Met-Arg-OH·H_2O	Boc-Met-ONp [14]	81	148 (methanol—ether)	—30 (*c* 1; DMFA)	0,67	0,37
XIV. Boc-Thr-Arg-OH·H_2O	Boc-Thr-OPcp [15]	75	182—192 (methanol—ether)*	—33 (*c* 1; DMFA)	0,50	0,32
XV. Z-Trp-Arg-OH·H_2O	Z-Trp-ONp [16]	75	162—164 (H_2O)	—32 (*c* 1; DMFA)	0,68	0,30
XVI. Z-*D*-Phe-Arg-OH·H_2O	Z-*D*-Phe-ONp [17]	91	169—171 (ethanol—ether)	—12 (*c* 1; DMFA)	0,64	0,38
XVII. Z-Ser-Arg-OH·0,5 H_2O	Z-Ser-OPcp [18]	93	134—135 (C_2H_5OH)*	—33 (*c* 1; DMFA)	0,52	0,26
XVIII. Boc-Asp(OBu^t)-Arg-OH·CH_3OH	Boc-Asp(OBu^t)-OTcp [19]	80	147—152 (methanol—ether)	Not. det.	0,67	0,33
XIX. Z-Abu-Arg-OH	Z-Abu-ONp [20]	85	135—145 (ethanol—ether)	—33 (*c* 1; DMFA)	0,65	0,36

*The peptide was preliminarily purified by preparative thin-layer chromatography on silica gel in system 2.

Deblockage of the Peptides (XI-XIV). To a solution of 1 mmole of a protected peptide in 2.5 ml of methanol was added 2 ml of trifluoroacetic or 2 ml of 4 N solution of HCl in dioxane, and the mixture was kept at room temperature for 1 h and was then evaporated in vacuum at 30°C, after which 3 ml of benzene was added to the residue and it was again evaporated to dryness. The final residue was dissolved in 5 ml of water and this solution was stirred with 5 ml of Amberlite IRA-410 in the OH^- form for 15 min; then the resin was filtered off and was washed three times with water, and the combined filtrates were lyophilized. Where necessary (purity check by TLC), the dipeptide was reprecipitated from methanol with ether.

Deblockage of the Peptides (XVI, XVII, and XIX). To a solution of 1 mmole of a protected peptide in 5 ml of acetic acid was added 5 ml of a 40% solution of hydrogen bromide in acetic acid, and the mixture was kept at room temperature for 1 h. Then it was diluted with ether and the product that precipitated was separated off and carefully washed on a glass filter with ether, after which it was dried in vacuum over KOH. Subsequent treatment with Amberlite IRA-410 was carried out as described above.

Deblockage of the Peptide (XV). A solution of 1 mole of the dipeptide in 5 ml of methanol was treated with 2 ml of cyclohexene and 0.2 g of palladium black, and the mixture was boiled for 1 h. After the separation of the catalyst, the solution was evaporated in vacuum, and the residue was reprecipitated from methanol with ether.

β-tert-Butyl Ester of Aspartyl Arginine. A solution of 1 mmole of the peptide (XIII) in 8 ml of 85% formic acid was kept for 1 h and was evaporated in vacuum at 30°C. The residue was treated with anion-exchange resin as described above.

The Tetrapeptides (I-IV) and (VI-X). A solution or suspension of 1 mmole of the appropriate deblocked dipeptide in 5 ml of DMFA was treated with 1.2 mmole of Dnp-Gly-Gly-ONp and the mixture was stirred at room temperature for 48 h with monitoring of the disappearance of the amino component. The solution was poured into a mixture of 100 ml of ethyl acetate and 50 ml of ether, and the resulting precipitate was carefully washed on the filter with ethyl acetate and ether. After drying in vacuum, the product was dissolved in 500-1000 ml of water, the solution was extracted 15-20 times with ethyl acetate, and the aqueous phase was lyophilized.

The Tetrapeptide Dnp-Gly-Gly-Asp-Arg-OH (V). One hour after the dissolution of 1 mmole of the tetrapeptide (IV) in 2 ml of trifluoroacetic acid the solution was diluted with ether, and the precipitate was filtered off, washed with ether, and dried in vacuum over KOH.

SUMMARY

Ten tetrapeptides of the general formula Dnp-Gly-Gly-X-Arg-OH have been synthesized by the activated ester method starting from arginine with an unprotected carboxy group.

It has been shown that the peptides obtained can be used to determine the activities of neutral and alkaline proteinases from various sources, and the most sensitive substrates, as a rule, proved to be the peptides with X = Phe, Met, and Abu.

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DETERMINATION OF THE GUANOSINE CONTENT OF TECHNICAL PREPARATIONS

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A method is proposed for the quantitative determination of the guanosine contents of technical preparations using the Folin reagent. The sensitivity of the method is 2.5 μg/ml, and the error of the determination does not exceed ±2.3%.

The purine and pyrimidine components of nucleic acids are widespread in nature. Derivatives of purine and pyrimidine enter into the composition of many coenzymes, vitamins, and antibiotics. A number of these valuable substances are obtained from technical preparations of the components of nucleic acids isolated from hydrolysates of them. Thus, an unpurified guanosine preparation of "technical" grade is the initial raw material for obtaining medicinal preparations (virazole [ribavirin] and thioguanine).

In the isolation of guanosine from hydrolysates of ribonucleic acid, preparations are formed which, in addition to guanosine, contain traces of other nucleosides – adenosine, uridine, cytidine – and also free guanine, proteins, and amino acids. Methods for analytical control are therefore required which permit guanosine to be determined in the presence of these impurities. The known methods of determining guanosine by UV spectroscopy [1] and potentiometric titration [2] are insufficiently selective. The chromatographic separation of the components of a technical preparation followed by UV spectrometric determination is laborious and has an extremely high error of determination (10-15%).

We have developed a method for the analysis of preparations of technical guanosine which permits the selective determination of the desired product in the presence of other nucleosides and also enables the presence of guanine, proteins, and amino acids to be allowed for. The determination is based on the capacity of guanine, unlike other nucleic bases, for reducing molybdotungstophosphoric acid (the Folin reagent) in an alkaline medium, as a result of which a colored compound with an absorption maximum at 770 nm is formed [3, 4].

To determine guanosine, the preliminary cleavage of the N-glycosidic bond is required, which is achieved by hydrolysis in 1 N hydrochloric acid on the boiling water bath for 10 min [5]. We have established the dependence of the intensity of absorption of the product of the interaction of guanine with the Folin reagent at 770 nm on its concentration in the solution undergoing photometry. The Lambert–Beer law is observed for guanine concentrations of 2.5-25 μg/ml. We first carried out the determination of guanosine in the presence of other nucleosides in artificial mixtures.

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