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SYNTHESIS AND CHARACTERIZATION OF AMINO PROTECTED PEPTIDES DERIVED FROM AMINO-α-HYDROXYIMINOPHOSPHONATES

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Abstract - Diisopropyl 2-amino-1-hydroxyiminoethylphosphonate (2a), derived from glycine, and diisopropyl 4-amino-1-hydroxyiminobutylphosphonate (2c), derived from GABA, were reacted with Boc-L-Phe-OH by the mixed anhydride method to yield novel peptide analogs diisopropyl 2-(t-Boc-L-Phe)amino-1-hydroxyiminoethylphosphonate (3a) and diisopropyl 4-(t-Boc-L-Phe)amino-1-hydroxyiminobutylphosphonate (3c). Diisopropyl 3-amino-1-hydroxyiminopropylphosphonate (2b), derived from β-Ala, and diisopropyl 2-amino-1-hydroxyiminopropylphosphonate (2d), derived from DL-Ala, were reacted with Boc-DL-Ala-OH by the mixed anhydride method to give peptide analogs diisopropyl 3-(t-Boc-L-Ala)amino-1-hydroxyiminopropylphosphonate (4b) and diisopropyl 2-(t-Boc-L-Ala)amino-1-hydroxyiminopropylphosphonate (4d) respectively. ^{1}H, ^{31}P NMR and FAB Mass spectra of the peptide analogs are reported.

INTRODUCTION

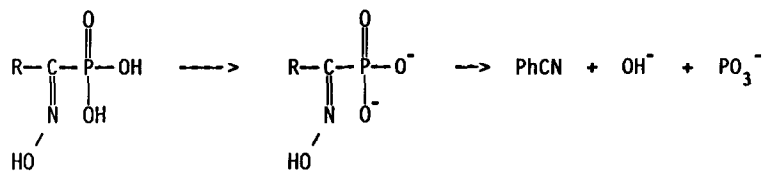
Phosphonate and phosphinate analogs of amino acids, either isolated from nature or obtained synthetically, display a wide range of interesting properties which may have considerable economic and clinical potential[1].

In addition to phosphaamino acids, peptides derived from them also show interesting activity mainly as antibacterials and inhibitors of proteases. Phosphapeptides synthesized and studied include compounds in which the phosphaamino acid replaces the amino acid residue at either the C-terminus or at the N-terminus. Most compounds of the former type are derivatives of 2-aminoethylphosphonic acid (P-Ala) and are of interest because of their potent antibacterial activity which results from the inhibition of some bacterial enzymes (Ala racemase and D-Ala:D-Ala ligase) involved in the synthesis of the bacterial cell wall[2]. In this series of bactericides the identity of the N-terminal amino acid appears to be of secondary importance and its role, presumably, is to determine the transport properties of the molecule across the bacterial cell wall[2].
On the other hand, peptides with N-terminal phosphaamino acids are characterized by the replacement of the normal peptide bond by a phosphoramidate group which, because of its tetrahedral structure, is viewed as an analog of the tetrahedral intermediate in the hydrolysis of the peptide bond. This concept stimulated the design and synthesis of a considerable number of "transition state analogs" to achieve selective inhibition of peptidases involved in essential regulating mechanisms such as angiotensin-converting-enzyme or enkephalinase[3].

Larger phosphapeptides of biological significance include tripeptides derived from phosphinothricine[4] that have significant herbicidal activity[5] and enkephalin analogs containing aminophosphonic residues at the C-terminus of pentapeptides[6].

In addition to peptides derived from phosphaamino acids mentioned above, there is considerable interest in other types of phosphorylated peptide derivatives. This interest stems from the recognition that enzymatic protein phosphorylations have been recognized in recent years as a major regulatory processes[7], and that their inhibitors (namely those of protein kinases) may be valuable anti-proliferative drugs[8].

One reason for our interest in α-hydroxyiminophosphonates (in the context of our continuing interest in acylphosphonic derivatives[9]) is their potential to serve as precursors to monomeric metaphosphate anion[10] or metaphosphate monoesters[11], and thus to act as phosphorylating agents. α-Hydroxyiminobenzylphosphonic acid (as an example) was shown previously in this laboratory to undergo facile fragmentation at physiological pH (equation 1)[10]. Consequently, it was postulated that this functional group offers the potential for the design of site specific biological phosphorylating agents, if by structural modifications, the transport and binding properties of the molecule can be suitably adjusted, to achieve affinity to specific sites[10].

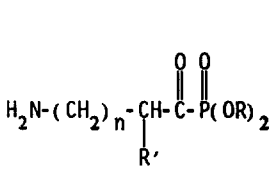


Equation 1

One of the goals of our ongoing research program is to design reagents that would be able to perform phosphorylation of a protein under physiological conditions. Another goal of our research is the design of novel phosphorus derivatives of peptides which will have affinity to the natural substrate of protein kinases and thus will block the sites of action of such enzymes. Since the phosphaacceptor sites of such substrates are highly populated by multiple basic amino acids[8], it seems conceivable that anions of hydroxyiminophosphonopeptides would have affinity to the phosphaacceptor site in the substrates of protein kinases. In addition, hydroxyiminophosphonates, because of their bidentate nature, have been shown to possess metal binding properties[12] and thus might yield new inhibitors of metalloenzymes, some of which are of medical importance.

Toward these goals, we decided to examine the possibility of incorporating amino-hydroxyiminophosphonates 2 described in our preceeding paper into peptides[13] Such compounds, in addition to their potential as biomimetic phosphorylating agents, mentioned above, may become potent inhibitors of metalloenzymes. Some of these enzymes are related to pathophysiological states

In the preceeding paper we described results from our studies concerning attempts to synthesize aminoacylphosphonates of type 1 and the corresponding oximes 2 derived from them[13]. Our results indicate that aminoacylphosphonates with unprotected amino and keto groups appear to be inherently unstable. In contrast, the corresponding oxime diester derivatives 2 could easily be prepared and isolated as the hydrochlorides.



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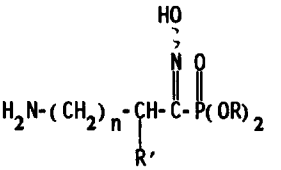


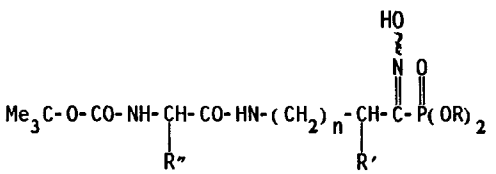
2, R = i-Pr

a, n = 0, R′ = H
b, n = 1, R′ = H
c, n = 2, R′ = H
d, n = 0, R′ = CH_3

RESULTS AND DISCUSSION

In this paper we wish to describe the preparation and the properties of such new types of phosphorus containing peptides. It was considered that since compounds 2 have two nucleophilic centers: the amino and hydroxyimino groups (possibly the latter being the better nucleophile), it might be necessary to protect the oxime hydroxy group to prevent it from undergoing acylation under coupling reaction conditions. On the other hand, it was also taken into account that in case the first acylation proceeds at the oxime hydroxy group, the products might behave as active esters, and therefore should be expected to react further to give the thermodynamically more stable peptides as the final products, either by intramolecular or intermolecular routes.

Aminohydroxyiminophosphonate hydrochlorides were obtained *via* the Arbuzov reactions[14] of the corresponding N-phthaloylaminoacyl chlorides with triisopropyl phosphite, followed by oximation of the keto groups and finally removal of the phthaloyl protecting group by hydrazine, as described previously[13]. The oximes 2a, 2c and 2d were single isomers of unassigned stereochemistry. ^{31}P nmr of 2b showed two absorptions 5.3 ppm (10%) and 0 ppm (90%) indicating the predominance of (Z) isomer[15].



3, R = i-Pr, R″ = Ph-CH_2

a, n = 0, R′ = H
c, n = 2, R′ = H

4, R = i-Pr, R″ = CH_3

b, n = 1, R′ = H
d, n = 0, R′ = CH_3

Acylations of aminohydroxyiminophosphonates 2 were carried out by the mixed anhydride method[16], employing an excess of the anydride generated by iso-butyl chloroformate, without protecting the oxime function. These reactions yielded peptides in good yields. It is not known however, whether there was any involvement of O-acyl oxime type compounds in these reactions.

In this work, four diisopropyl amino-α-hydroxyiminophosphonates (2a-d) were incorporated into peptide units. The aminohydroxyiminophosphonates[13] derived from glycine (2a) and from γ-aminobutyric acid (2c) were reacted with mixed anhydride derived from t-butyloxycarbonylphenylalanine (Boc-Phe) to yield peptides 3a and 3c, while aminohydroxyiminophosphonates derived from β-alanine (2b) and alanine (2d) were reacted with mixed anhydride derived from t-butyloxycarbonylalanine (Boc-Ala) to yield peptides 4b and 4d.

There were no attempts made to optimize yields of the peptide analogs 3 and 4, which were in the range of 40 - 50%. Products 3 and 4 were characterized by elemental analyses and NMR spectra (^{1}H and ^{31}P) which were consistent with the structures. Amino acid analyses of 3a and 3c yielded, in addition to the expected Phe, also the parent amino acid of the hydroxyiminophosphonic moiety (GABA and Gly). This indicates that during the hydrolysis of the sample which preceded the chromatographic analysis these hydroxyiminophosphonate esters were transformed to the parent compound. In contrast, amino acid analysis of 4d gave, instead of two equivalents of Ala, one equivalent of Ala and a peak appearing as His resulting, presumably, from the 2-amino-1-hydroxyiminophosphonate moiety.

In addition to these methods of characterization, our compounds were subjected also to fast atom bombardment mass spectrometry (FAB-MS). The characteristic $[M+H]^{+}$ quasimolecular ions and fragmentation patterns obtained by this method provide further support for the assigned structures of the coupling products as the anticipated peptides derived from the N_{α}-protected amino acid and the diisopropyl amino-α-hydroxyiminophosphonates. Fragmentations, which involve both amino- and phosphorus-terminal protecting groups, were observed for all four peptide analogs 3a, 3c, 4b, and 4d.

One of the most abundant peaks in all four mass-spectra is $[MH-Boc]^{+}$ resulting from the splitting off the N_{α}-Boc protecting group, which is followed by the consecutive cleavages of the isopropyl groups, leading eventually to the release of H_3PO_4 with the creation of a nitrile end group. Another characteristic point of cleavage is the peptide bond. The variety of fragments observed from the cleavage of this bond include both phosphonate containing (diesterified or mono- or bisdealkylated) and the dephosphonylated species.

There is a certain resemblance between our results and the fragmentation pattern that has been described for phosphates and phosphonates under electron impact ionization. These also split off the ester alkyl groups with concomitant H transfer, forming either $[R\text{-}P(O)OH(OR)]^{+}$ or $[R\text{-}P(O)(OH)_2]^{+}$ ions (17). Similar behavior was also noted in the chemical ionization mass spectra of phosphate pesticides (18).

CONCLUSION

The work described demonstrates the possibility for the introduction of hydroxyiminophosphonic moieties as building blocks in the design of novel peptide analogs that might lead to potential bioactive agents such as inhibitors of proteolytic enzymes, in-situ phosphorylating agents, in-vivo calcium chelators and metal sequestering agents.

EXPERIMENTAL SECTION

General

Elmental microanalyses was carried out at the Microanalytical Laboratory of The Hebrew University of Jerusalem. Nuclear magnetic resonanace spectra were obtained on a Bruker WH-300 or on a Varian VXR-300S instrument. Chemical shifts are reported in ppm downfield from TMS as internal standards in ^{1}H spectra and from 85% orthophosphoric acid as external standard in ^{31}P spectra. Positive chemical shifts are at low field with respect to the standard. Amino acid analyses were obtained on LKB-4400 amino acid analyzer equipped with a Spectra-Physics SP-4100 printer-plotter computing integrator using 4-component sodium buffer systems and a standard 70-min program. Hydrolyses of the peptide samples for amino acid analysis were carried out on 1 mg samples in constant-boiling HCl (0.5 mL), which was degassed, sealed under high vacuum, and heated at 110°C for 20 h. The hydrolysate was dried under vacuum over KOH pellets and diluted with 0.2 N sodium citrate buffer (0.2 mL), pH 2.2. All mass spectrometric measurements were carried out using a Vacuum Generator (VG) Model 70 VSEQ mass spectrometer, equipped with Cs fast ion gun operated at about 35kV. Glycerol was used as the liquid matrix to introduce the sample into the probe. The purity of these peptides was checked by HPLC at room temperature, using a Merck Hitachi Model L-6200 intelligent pump with RP-8 or RP-18 Hibar Lichrospher RP columns (Merck), flow rate 1 mL/min. The eluted peaks were monitored at 254 nm. DMF was distilled from P_2O_5 under reduced pressure, then redistilled from ninhydrin. THF was distilled from CaH_2 under nitrogen.

Diisopropyl 2-(tert-butyloxycarbonyl-L-phenylalanylamino)-1-hydroxyiminoethylphosphonate (3a). This compound was synthesized from Boc-L-Phe-OH and 2a.HCl by the method described for compound 3c with the following modifications: DMF as solvent was replaced with dry THF, and the crude product was chromatographed on a Lichroprep C8 column (2.5x50 cm) using a MeOH/water gradient (from 40-50% MeOH in water in 2 hrs) and eluted with 45% methanol in water. The major peak was pooled and lyophylized to give the semisolid product. Yield: 39%. HPLC (RP-8) MeOH/H_2O (70/30), t_R = 5.32 min. NMR ($CDCl_3$) ^{1}H: 7.24 (5H, m), 7.18 (1H, bs), 5.34 (1H, bd), 4.70 (2H, m) 4.25 (3H, m), 3.36 (2H, m), 1.30 (21H, m). ^{31}P: 1.63 ppm. FAB-MS (m/z): 486 $(M + H)^+$, (68%); 470, (6%); 414, (7%); 386, (30%); 344 (18%); 302, (22%); 204 , (22%); 120, (100%); 91, (11%). Anal. Calcd. for $C_{22}H_{36}O_7N_3P$: C, 54.43; H, 7.42; N, 8.61; P, 6.39. Found: C, 54.07; H, 7.52; N, 8.61, P, 6.73. Amino acid analysis: Phe, 1.01, Gly, 1.00.

Diisopropyl 4-(tert-butyloxycarbonyl-L-phenylalanylamino)-1-hydroxyiminobutylphosphonate (3c). NMM (0.44 mL, 4.0 mMol) followed by IBCF (5.2 mL, 4.0 mMol) were added to a solution of Boc-L-Phe-OH (1.062 g, 4.0 mMol) in dry DMF (7.5 mL). To this mixture was then added a solution of diisopropyl 4-amino-2-hydroxyiminobutylphosphonate hydrochloride (2c.HCl, 0.98 g, 3.2 mMol) and NMM (0.4 mL, 3.65 mmol) in dry DMF (7.3 mL), and the coupling was allowed to proceed at -15°C under nitrogen for 2 h, then at room temperature overnight. A solution of 2N $KHCO_3$ (6 mL, 12 mMol) was added to the reaction mixture at 0°C and stirring was continued for 30 min. The solvent was removed in vacuo, the residue was taken up in a mixture of AcOEt (75 mL) and water (25 mL) and the organic phase was washed successively with 2N $KHCO_3$ (25 mL), brine (25 mL), 1N $KHSO_4$ (25 mL), brine (25 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness in vacuo. The crude product (1.55 g) was chromatographed on an LPLC silica gel column (2.5x40 cm) and eluted with 50% AcOEt in hexane. Evaporation of the solvent in vacuo yielded 0.83 g (50%) of semisolid product. HPLC: (RP-18) MeOH/H2O (70/30), t_R = 6.23 min. NMR ($CDCl_3$) ^1H: 7.23 (5H, m), 6.9 (1H, bs), 5.4 (1H, bs), 4.70 (2H, m) 3.23 (1H, m), 3.12 (4H, m), 2.39 (2H, m), 1.72 (2H, m), 1.37 (9H, s), 1.30 (12H, m). ^{31}P: 3.026 ppm. FAB-MS (m/z): 514 $(M + H)^+$, 80%; 500, 57%; 414, 57%; 277, 33%; 232, 28%; 165, 24%; 149, 48%; 120, 100%. Anal. Calcd. for $C_{24}H_{40}O_7N_3P$: C, 56.14; H, 7.79; N, 8.18; P, 6.04. Found: C,55.86; H,7.90; N,8.08; P,5.89. Amino acid analysis: GABA, 1.00; Phe, 1.08.

Diisopropyl 3-(tert-butyloxycarbonyl-L-alanylamino)-1-hydroxyiminopropylphosphonate (4b). This compound was synthesized, from Boc-DL-Ala-OH and 2b.HCl, in 50% yield using the method described for compound 3c. HPLC (RP-8) MeOH/H_2O (70/30), t_R = 3.82 min. NMR ($CDCl_3$) ^1H: 4.74 (2H, m); 4.10 (1H, m); 3.47 (2H, m); 2.75 (2H, m); 1.43 (12H, m); 1.33 (12H, m); ^{31}P: 7.69 (m, 13%) and 4.08 (m, 87%). MS (m/z): 423, 367, 350, 323, 308, 279, 264, 238, 213, 196, 168, 165, 139. FAB MS (m/z): 424 $(M + 1)^+$, (84%); 408, (17%); 353, (18%); 324, (100%) ; 282, (15%); 240, (10%); 142, (38%). Anal. Calcd. for $C_{17}H_{34}N_3O_7P$: C, 48.00; H, 8.00; N, 9.88; P, 7.32. Found: C, 48.08; H, 8.12; N, 9.58; P, 7.30.

Diisopropyl 2-(tert-butyloxycarbonyl-L-alanylamino)-1-hydroxyiminopropylphosphonate (4d). This compound was synthesized from Boc-DL-Ala-OH and 2d.HCl, in 51% yield using the method described for compound 3c, except that DMF was replaced with THF. HPLC (RP-18) MeOH/H_2O (70/30), t_R = 3.87 min. ^1H-NMR ($CDCl_3$): 7.47 (1H, m), 5.51 (1H, m), 5.31 (1H, m), 4.62 (2H, m), 4.14 (1H, m), 1.36 (9H, s) , 1.26 (18H, m). FAB-MS (m/z) 424 $(M + H)^+$, (100%); 408, (36%); 368, (26%); 353, (9%); 324, (18%). Anal. Calcd. for $C_{17}H_{34}N_3O_7P$: C, 48.00; H, 8.00; N, 9.88; P, 7.32 Found· C, 48.04; H, 8.14; N, 9.38; P, 7.32. Amino acid analysis: Ala, 1.00 and a peak appearing as His.

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