hydride abstraction, in competition with dehydrogenation. For higher alkanes, it is demonstrated that hydride abstraction by Au^+ generates internally excited alkyl cations which fragment in competition with collisional stabilization. Generation of the reactive gold carbene, $AuCH_2^+$, from methyl halides, methide, and hydride abstraction studies allows estimation of lower limits for Au^+–CH_2, Au–CH_3, and Au–H bond dissociation energies (D-(Au^+–CH_2) ≥ 95.0 kcal/mol, D(Au–CH_3) ≥ 45.8 kcal/mol, and D(Au–H) > 67 kcal/mol). In contrast to gold cation, Au^- is relatively inert and in the present study its reactions have been limited to halide displacement and gold–dihalide anion formation. Generation of $AuCBr_2^-$ from $CHBr_3$ appears to be the first reported gas-phase anionic metal carbene. The apparent unreactivity

of Au^- may be due to its stable $d^{10}s^2$ electronic configuration.

Liquid Secondary Ionization Mass Spectrometric Characterization of Two Synthetic Phosphotyrosine-Containing Peptides

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Abstract: *N*-(*tert*-Butoxycarbonyl)-*O*-(dibenzylphosphono)-L-tyrosine (1), a suitably protected phosphotyrosine, has been prepared for peptide synthesis. Compound 1 and its intermediate precursors were characterized by elemental analyses, liquid secondary ion mass spectrometry (LSIMS), and NMR spectral data. With use of 1, both a tri- and a tetrapeptide, Val-Tyr(PO_3H_2)-Phe and Arg-Tyr(PO_3H_2)-Val-Phe, were prepared via solid-phase synthesis and fully characterized by LSIMS. LSIMS spectra were taken in both positive and negative ion modes as well as metastable decomposition spectra of the molecular ions of the two peptides with a B/E linked scan. For comparison, similar LSIMS spectra were acquired for peptides containing a phosphoserine (kemptide) and sulfated tyrosine (caerulein and cholecystokinin-8). The most significant features of the mass spectra of the phosphotyrosine-containing peptides are abundant molecular ions in both the positive and negative ion mode and peaks corresponding to losses of HPO_3 (−80 mu) and HPO_4 (−96 mu) from the parent molecules. Peaks for HPO_3 and HPO_4 loss are of very low abundances in peptides containing phosphoserine. The elemental compositions of the peaks corresponding to the losses of HPO_3 and HPO_4 from the parent molecular ions were confirmed by accurate mass measurements. The losses of HPO_3 and HPO_4 from the parent molecules of these two phosphotyrosine-containing peptides are important because they allow a distinction to be made between them and peptides containing phosphoserine or sulfated tyrosine (e.g., caerulein and cholecystokinin-8), the latter of which undergo much more extensive SO_3 loss (−80 mu) in the positive ion mode than the HPO_3 loss observed for the two phosphotyrosine-containing peptides.

Recently, a number of transforming proteins originating from the oncogenes of avian and mammalian tumor viruses have been characterized and shown to possess tyrosine-specific protein kinase activity.[1] Tyrosine kinase activity is also present in the receptors for epidermal growth factor (EGF)[2] insulin[3] and platelet derived growth factor[4] as well as in a number of proto-oncogenes.[5] In fact, it appears that several of these retroviral transforming proteins are related in structure to the growth factor receptors. In the case of the viral erb-B oncogene product, a high degree of homology is found with the intracellular kinase domain of the EGF receptor, while a corresponding extracellular domain appears to be absent.[6,7]

Although these enzymes are known to mediate phosphoryl transfer between ATP and tyrosine residues in endogenous cytoplasmic proteins, their mechanism of action is not clearly understood. Neither are the consequences of the enzymatic phosphorylation of these proteins known or the effect, if any, that peptides resulting from proteolytic degradation of these phosphorylated proteins have on cellular processes. However, phosphorylations of specific amino acids in numerous enzymes, receptors, and ion channels are known to be important regulators of their respective functions. In this respect, it is essential to determine the substrate specificity for phosphorylation, which first requires the isolation and analysis of phosphopeptides. Although our understanding of the substrate specificity for tyrosine kinases is lacking, inhibitors based on the enzyme substrates would also be desirable, as tyrosine-specific protein kinases are enzymes that

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represent the only accessible chemical mechanism for the transformation encoded by the oncogenes of retroviruses.

Analytical techniques for the identification and sequence analysis of phosphotyrosine-containing peptides need to be improved both for the analysis of phosphopeptides generated from these larger proteins by enzymatic hydrolysis or, as in our case, for the direct analysis of synthetic peptide substrates acting as potential inhibitors of the tyrosine kinases. Currently, ^{32}P-phosphotyrosine is identified either after partial acid hydrolysis by a combination of electrophoresis[7] and TLC[8] or in sequence analysis as released radioactivity in conjunction with PTH-phosphotyrosine determination by TLC.[9] In gas-phase Edman degradation, PTH-phosphotyrosine cannot be detected and either a spinning cup or solid-phase sequencing approach must be used.[10] Even in the latter methods the poor extractability of PTH-phosphotyrosine results in severe carryover into subsequent cycles and can obscure additional phosphorylation sites, if present. This is also true for phosphoserine and phosphothreonine, which undergo β-elimination of phosphate under basic conditions to complicate further their identification and quantitation. Poor extractability and high carryovers are also a problem for analyzing radiolabeled inorganic phosphate from eluates of the spinning cup and solid phase sequencing filter after each Edman cycle. Recently, a method for analyzing radiolabeled phosphate directly from the supporting filter for a gas-phase sequencer has been proposed[11] but requires that the sample filter be cut between cycles followed by extraction and HPLC analysis.

Clearly, a method that could detect nonradioactive phosphotyrosine in peptides and be free of the problems of PTH-phosphotyrosine identification in cyclical Edman-based methods is needed. In this paper, we describe the application of liquid secondary ionization mass spectrometry (LSIMS)[12] to the analysis of two new synthetic phosphotyrosine-containing peptides. For this purpose, we have synthesized N-(tert-butoxycarbonyl)-O-(dibenzylphosphono)-L-tyrosine (1), a suitably protected phosphotyrosine for peptide synthesis. To date, only two synthetic methods for producing phosphotyrosine-containing peptides have been reported: one for N-terminal phosphotyrosine,[13] and a more recent synthesis[14] involving the use of an analogous dimethyl-substituted phosphotyrosine derivative.

Compound 1 was incorporated by solid-phase peptide synthesis into a tri- and a tetrapeptide, Val-Tyr(PO$_3$H$_2$)-Phe and Arg-Tyr(PO$_3$H$_2$)-Val-Phe, respectively, using a Merrifield resin.[14] LSIMS spectra were obtained for both peptides in the positive and negative ion mode; in addition, B/E linked scans were used to identify gas-phase fragmentation products of the protonated molecules. The spectra obtained indicate that these peptides can easily be distinguished from their dephospho forms by nominal molecular weight and, in addition, appear to undergo cleavage of the phosphate moiety to an appreciably higher extent than peptides containing phosphoserine.

Experimental Section

N-(tert-Butoxycarbonyl)-L-tyrosyl p-Nitrobenzyl Ester (2). Boc-L-tyrosine (14.06 g, 0.05 mol) and triethylamine (14 mL, 0.1 mol) were

(8) Symbols for amino acids and peptides and protection groups are in accordance with IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations used: SPPS, solid phase peptide synthesis; HPLC, high-performance liquid chromatography; CMC, carboxymethylcellulose; LSIMS, liquid secondary ion mass spectrometry; TLC, thin-layer chromatography; PTH, phenylthiohydantoin; CCK-8, cholecystokinin-8. All amino acids are of the L configuration.

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dissolved in 150 mL of EtOAc with stirring at 80–90 °C in a drierite-protected round-bottom flask equipped with a reflux condenser. The mixture was then cooled before p-nitrobenzyl bromide (21.6 g, 0.1 mol) was added in a single portion. The mixture was heated to 80–90 °C, again with stirring. The solid quickly dissolved before a white precipitate of triethylamine·HBr was formed. The reaction was stirred overnight at 80–90 °C and the solid was removed by filtration and washed with EtOAc. The combined filtrates were washed with 1 N HCl, cold water, 5% NaHCO$_3$, and cold water. The solution was then dried with MgSO$_4$ and evaporated to give an oil. The oil was dissolved in 100 mL of EtOAc/hexane (3:7, v:v) to induce crystallization. The solid was recrystallized with EtOAc/hexane (1:4, v:v). A quantitative yield was obtained (mp 111 °C, uncorrected). TLC (silica gel) with EtOAc/hexane (1:1, v:v) gave an R_f value of 0.58.

Anal. Calcd for C$_{21}$H$_{24}$N$_2$O$_7$: C, 60.57; H, 5.81; N, 6.72. Found: C, 60.39; H, 5.80; N, 6.66.

^{1}H NMR (CDCl$_3$–TMS) δ 8.21–7.29 (pair of doublets, 4 H), 6.99–6.63 (pair of doublets, 4 H), 5.18 (s, 2 H), 5.00 (d, 1 H), 4.50 (q, 1 H), 2.99 (d, 2 H), and 1.41 (s, 9 H).

^{13}C NMR (CDCl$_3$–TMS) δ 171.95, 155.45, 147.79, 142.37, 130.32, 128.54, 127.11, 123.47, 115.64, 80.6, 65.49, 54.95, 37.60, 28.30.

N-(tert-Butoxycarbonyl)-O-(dibenzylphosphono)-L-tyrosyl p-Nitrobenzyl Ester (3). In a round-bottom flask triethylamine (8.03 mL, 58 mmol) was added dropwise to a mixture of **2** (16 g, 38 mmol), dibenzyl phosphite (15.12 g, 58 mmol), and CCl$_4$ (100 mL) in 100 mL of EtOAc. This procedure is adapted from the general method of Steinberg.[16] The reaction was monitored by TLC–silica gel with EtOAc/hexane (1:1, v:v), R_f 0.48. After 3 h of stirring, more portions of both dibenzyl phosphite (15.12 g) and Et$_3$N (8.03 mL) were added, and the reaction was continued overnight. The mixture was diluted with EtOAc, filtered to remove Et$_3$N·HCl, washed with cold HCl (1 N) and 5% NaHCO$_3$, dried with MgSO$_4$, and evaporated to an oil. To this oil were added CCl$_4$ (50 mL), dibenzyl phosphite (7.31 g), and triethylamine (4.05 mL). The reaction mixture was then stirred overnight until no trace of starting material remained. The mixture was again diluted with EtOAc, filtered, washed, dried, and evaporated to an oil as described above. This prepurified oil was then subjected to a silica gel flash column (Merck grade 60, 230–400 mesh, Aldrich). The commercial silica gel was activated and dried by heating to 150 °C in a beaker for a few hours and then stored in a vacuum desiccator overnight. The column was prewashed with hexane before the compound, dissolved in a minimum amount of EtOAc, was added to it. The compound was washed with hexane before being eluted with EtOAc/hexane (4:6, v:v). The solvents were evaporated and the remaining oil was diluted with 200 mL of EtOAc/hexane (3:7, v:v) for crystallization. A quantitative yield of the product was collected after filtration (mp 81–82 °C). It was then recrystallized from EtOAc/hexane (3:7, v:v).

Anal. Calcd for C$_{35}$H$_{37}$N$_2$O$_{10}$P: C, 62.13; H, 5.51; N, 4.14; P, 4.58. Found: C, 61.99; H, 5.49; N, 4.10; P, 4.6.

^{1}H NMR (CDCl$_3$–TMS) (GN-500 MHz instrument) δ 8.20–7.37 (pair of doublets, J = 8.5 Hz, 4 H), 7.33 (m, 10 H), 7.04 (s, 4 H), 5.18 (s, 4 H), 5.15 (pair of singlets, 4 H), 6.06 (d, 1 H), 4.61 (quartet, J = 6.45 and 14.49 Hz, 1 H), 3.06 (d, J = 6.26 Hz, 2 H), and 1.42 (s, 9 H).

^{31}P NMR (CDCl$_3$ with 0.85% H$_3$PO$_4$ in D$_2$O as an external standard) δ −6.36 which shows a singlet in a proton-decoupled and a quintet (J = 8.4 Hz) in a proton-coupled NMR spectrum (lit.[17] 6.3, s).

N-(tert-Butoxycarbonyl)-O-(dibenzylphosphono)-L-tyrosine (1). A mixture of Na$_2$S$_2$O$_4$ (5.45 g, 31.33 mmol) and Na$_2$CO$_3$ (3.32 g, 31.33 mmol) in 25 mL of boiled water was added to a stirred solution of **3** (5.3 g, 7.83 mmol) in 50 mL of CH$_3$CN.[17] The reaction mixture was stirred vigorously at 50–60 °C for 1 h and then 2 h at room temperature. It was cooled in an ice-water bath and acidified to pH 3–4 with 1 N HCl. The product was extracted with ether, and the ether solution was washed with cold HCl (0.5 N), dried over MgSO$_4$, and evaporated down to an oil. The product was then extracted with 5% NaHCO$_3$, and the base extract was washed with ether (200 mL) and EtOAc (50 mL). It was then cooled before acidification to pH 3–4 with 1 N HCl. The final product was extracted with ether and the solvent was dried with MgSO$_4$ before evaporation to give a light yellow oil. The oil solidified after adding ether/hexane. The solid was collected by filtration (approximately 70% yield). It was recrystallized from ether/hexane (2:8, v:v) (mp 91.5–92.5 °C, uncorrected). TLC (silica gel) with EtOAc/hexane (5:5, v:v) gave an R_f value of 0.22.

Anal. Calcd for C$_{28}$H$_{32}$NO$_8$P: C, 62.10; H, 5.96; N, 2.59; P, 5.72. Found: C, 61.92; H, 6.01; N, 2.60; P, 5.75.

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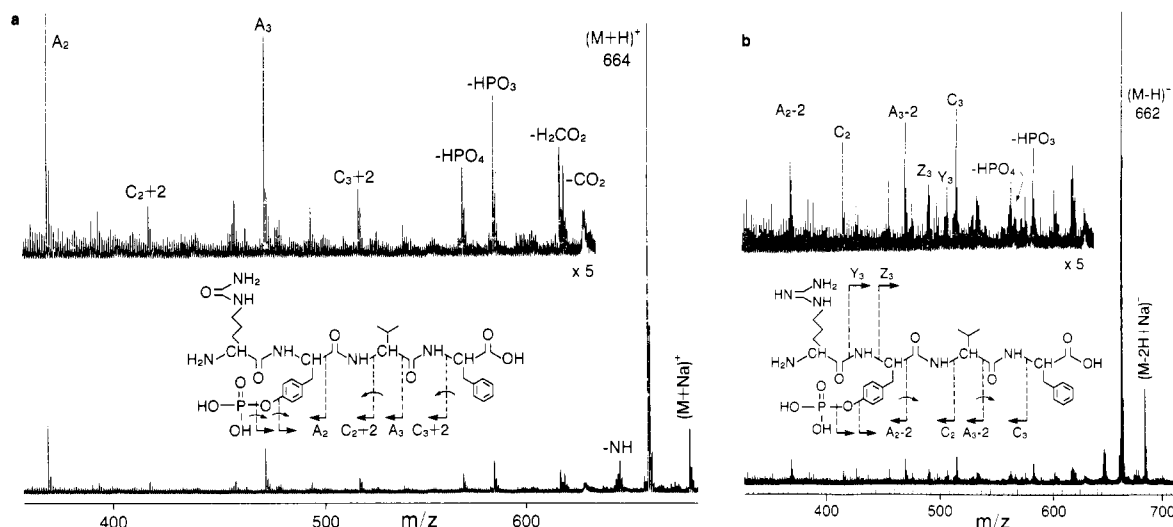(17) Guibe-Jampel, E.; Wakselman, M. *Synth. Commun.* **1982**, *12*, 219–223.

Figure 1. LSIMS spectra of tetrapeptide Arg-Tyr(PO_3H_2)-Val-Phe: (a) positive ion mode and (b) negative ion mode.

^{31}P NMR (240 MHz) (CDCl$_3$ with 0.85% H$_3$PO$_4$/D$_2$O as an external standard) showed a singlet at -6.79 ppm for a proton-decoupled and a quintet (J = 8.4 Hz) at -6.79 ppm for a proton-coupled spectrum (lit.[17] 6.6, s).

^{1}H NMR (CDCl$_3$-TMS) (GN-500 MHz instrument) δ 7.33-7.29 (m, 10 H), 7.11-7.03 (pair of doublets, J = 8.05 Hz, 4 H), 5.11 (d, J = 8.4, 4 H), 5.07 (s, 1 H), 4.60 (m, 1 H), 3.16-3.05 (pair of doubled doublets, 2 H), 1.42 (s, 9 H).

Synthesis of Tyr(PO_3H_2)-Containing Peptides. Protected amino acids (Boc-Arg(Tos)-OH, Boc-Phe-OH, Boc-Val-OH) were purchased from Penninsula Laboratories. Boc-Phe-OH was coupled to chloromethylated resin (Merrifield Polymer FLUKA, polystyrene - 1% divinylbenzene, 200-400 mesh, 0.67 mMol Cl/g) by the cesium salt procedure[15] which yielded 0.35 mmol of amine/g (determined by the picric acid method[18]).

The peptides were synthesized on the solid phase[19] by starting from 0.5 g of Boc-Phe-O-Resin and using the Yamashiro et al.[20] SPPS cycle protocol. After the last amino acid had been coupled, the N-terminal Boc-protection was removed with TFA and the peptides were cleaved with a mixture of 9 mL of liquid HF[21] and 1 mL of anisole at 0 °C for 30 min. The HF was evaporated with a stream of nitrogen (10 min) and the residues were dried over NaOH pellets in vacuum (about 2 h). The dry residues were then washed with ice cold ether and the peptides were extracted with 0.5 M AcOH (10 mL).

The peptides were subjected to gel filtration on a Sephadex G-10 column equilibrated with 0.5 M AcOH. The peptide-containing fractions were collected and after lyophilization they were purified on a CMC column equilibrated with 0.01 M AcONH$_4$. The elutions were done with a gradient formed through a constant 500 mL mixing volume by 0.1 M AcONH$_4$. The phosphotyrosine-containing fractions for the tri- and tetrapeptide (5-10% relative amounts by weight) were further purified by HPLC with use of reverse phase C$_{18}$ analytical column (Phase Separations, U.K.) and eluting the peptides with a linear gradient (1%/min) of acetonitrile starting with 100% H$_2$O containing 0.1% trifluoroacetic acid.

LSIMS of Val-Tyr(P)-Phe and Arg-Tyr(P)-Val-Phe. Phosphopeptides isolated by HPLC and/or liquid chromatography on CMC were dissolved on the probe tip in approximately 2 μL of glycerol or thioglycerol-glycerol (1:2) with approximately 1-10 μg of each peptide. Commercial peptides containing sulfated tyrosine (caerulein and CCK-8, Sigma) and phosphoserine (kemptide, gift from P. Cohen) were first checked for purity by HPLC (ca. 99% pure) and then analyzed by LSIMS in the positive and negative ion modes. Approximately 3-4 μg of each peptide was dissolved in either glycerol or thioglycerol-glycerol (1:2) and applied to the probe tip in an identical manner to the phosphotyrosine peptides. Mass spectra were obtained with a Kratos MS-50S double focusing mass spectrometer equipped with post-acceleration detection and a Cs$^+$ ion LSIMS ion source designed in this laboratory.[22]

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The Cs$^+$ primary beam energy was approximately 8 keV. A scan rate of 300 s/decade was used and the resulting spectra were recorded on a Gould ES-1000 electrostatic recorder.

Results

The protected phosphotyrosine derivative *N*-(*tert*-butoxycarbonyl)-*O*-(dibenzylphosphono)-L-tyrosine was obtained in high yield as a white crystalline material (mp 91.5-92.5 °C). In contrast, Valerio et al.[14] using a similar synthetic route had described it as a "light yellow oil" and had characterized it only by ^{31}P NMR and IR spectroscopy. The product is soluble in dichloromethane and other organic solvents which satisfies solubility requirements for normal solid-phase peptide synthesis. The identity of this product was confirmed by elemental analysis and both ^{1}H NMR (500 MHz) and ^{31}P NMR (90 MHz). A LSIMS analysis indicated the presence of a single protonated molecule (MH$^+$) at m/z 542, in agreement with the predicted molecular weight of 541. From these data, compound 1 was judged to be suitable for direct use for BOC solid-phase peptide synthesis.

Two syntheses were attempted incorporating *O*-(dibenzylphosphono)-L-tyrosine into the peptide chain: the tripeptide, Val-Tyr(PO_3H_2)-Phe, and the tetrapeptide, Arg-Tyr(PO_3H_2)-Val-Phe. After deprotection with anhydrous HF, the CMC separations indicated that several peptide products were formed in each synthesis. LSIMS analysis of the reaction mixture prior to CMC separations also showed the presence of more than one protonated molecule. Further LSIMS analysis of the CMC fractions showed that the tripeptide Val-Tyr(PO_3H_2)-Phe was present in fractions 9-10 as the major component in low yield. This fraction was separated by reverse-phase HPLC and the major component, Val-Tyr(PO_3H_2)-Phe, was again analyzed by LSIMS. The tetrapeptide Arg-Tyr(P)-Val-Phe was also shown by LSIMS analysis to be impure and required further purification by both CMC and reverse-phase HPLC. The identities of the major side products were deduced from the LSIMS spectra, primarily from their corresponding molecular weights. In both syntheses these were identified as dephospho analogues and peptides containing a benzyl and/or a trifluoroacetyl moiety, sometimes in addition to a phosphoryl group on the tyrosine. At present, it appears that the deprotection scheme is the source of the majority of side reactions, and not the incorporation of the dibenzyl-protected BOC-phosphotyrosine residue into the peptide chain. We are presently investigating several modifications to the deprotection scheme that will either reduce or eliminate these unwanted side reactions before larger peptides are synthesized as protein kinase inhibitors. Nevertheless, both synthetic preparations gave sufficient quantities of the desired tri- and tetraphospho-peptide so that they could be easily separated from the dephosphopeptides and other impurities and their mass spectrometric properties determined by LSIMS.

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The positive LSIMS spectrum of the tetrapeptide Arg-Tyr-(PO_3H_2)-Val-Phe shown in Figure 1a gave an abundant protonated molecular (MH^+) at m/z 664. Sequence ions typical of small peptides were also observed with considerably lower abundances. These corresponded to cleavage between the amide nitrogen and the α-carbon with a proton transfer to form the protonated N-terminal amide ions at m/z 516 ($C_3 + 2$)[23] and 417 ($C_2 + 2$). In addition, the even more abundant N-terminal alkyl ions at m/z 471 (A_3) and 372 (A_2) were present. These ions are often enhanced for peptides containing an N-terminal arginine. Lower mass ions that would be expected to result from subsequent cleavage of the phosphotyrosine residue to produce ions at m/z 174 ($C_1 + 2$) and 129 (A_1) were not observed. This lower mass region (below m/z 350) is contaminated by matrix and HPLC related impurities and may have obscured these latter sequence ions.

No fragment ions were observed in the positive ion LSIMS spectrum of Arg-Tyr(P)-Val-Phe which would have involved charge retention on the C-terminus. This is presumably due to the presence of arginine at the N-terminus which would be expected to be the major site of protonation and charge retention following fragmentation.

The fragment ions originating from loss of phosphate from tyrosine are by far the most important since this is the structural feature of interest. Ions at m/z 584 and 568 were observed with an intensity approximately 5–10% of the molecular ion corresponding to loss of HPO_3 and HPO_4, respectively, from the MH^+. These assignments were confirmed by accurate mass measurements to within 1.5 ppm, i.e., 584.3203 ($MH^+ - HPO_3$, m/z calcd = 584.3196) and 568.3249 ($MH^+ - HPO_4$, m/z calcd = 568.3247). Other fragment ions resulting from small neutral losses could also be readily assigned as deamination ($-NH_2$, m/z 648), decarboxylation ($-CO_2$, m/z 620), and loss of formic acid ($-H_2CO_2$, m/z 618). In addition, a small metastable decomposition (loss of OH or NH_3) results in a broad peak with a maximum at m/z 630.

The negative ion LSIMS spectrum of the tetrapeptide (see Figure 1b) was very similar to the positive ion spectrum, although the fragment ions were less abundant. An abundant $(M - H)^-$ ion was present at m/z 662, as were sequence ions corresponding to C_3, $A_3 - 2$, C_2, and $A_2 - 2$ (m/z 514, 469, 415, and 370, respectively), but two fragment ions with charge retention at the C-terminus were also observed. These latter ions correspond to the loss of arginine via cleavage at either side of the neighboring amide nitrogen giving rise to the deprotonated C-terminal amine ion at m/z 506 (Y_3) and C-terminal alkyl ion at m/z 490 ($Z_3 - 1$).

As was the case in the positive ion LSIMS spectrum, fragments arising from losses of HPO_3 (m/z 582) and HPO_4 (m/z 566) from phosphotyrosine were present, although not so abundant. As might be expected for negative ion LSIMS, a significant ion at m/z 97 ($H_2PO_4^-$) was also observed. The identity of the $H_2PO_4^-$ ion was confirmed by accurate mass measurement to within a few ppm (exptl 96.972, calcd H_2PO_4 96.969).

The positive and negative ion LSIMS spectra of the synthetic tripeptide Val-Tyr(PO_3H_2)-Phe were very similar to those obtained for the phosphorylated tetrapeptide. The positive ion spectrum (Figure 2) contained a prominent MH^+ 508 as well as a cationated specie at m/z 530 (MNa^+). Sequence ions were also observed at m/z 409 ($Y_2 + 2$) corresponding to loss of neutral valine from the N-terminus and at m/z 360 ($C_2 + 2$) and 343 (B_2) which result from loss of (neutral) phenylalanine from the C-terminus. Fragment ions were also observed at m/z 428 (–80 mu) and 412 (–96 mu) of approximately 4% of molecular ion abundance which confirms the tendency for loss of HPO_3 and HPO_4 from the MH^+ ion. The negative ion LSIMS spectrum also contained somewhat weaker peaks corresponding to losses of 80 and 96 mu, approximately 1–2% of the molecular ion abundance. Sequence ions in
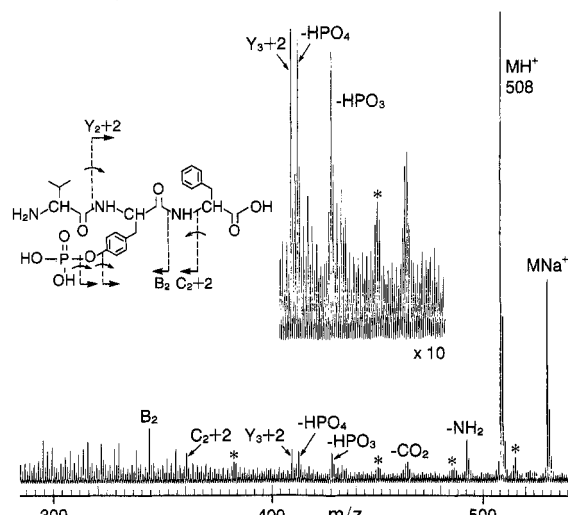

Figure 2. Partial LSIMS spectra of tripeptide Val-Tyr(PO_3H_2)-Phe in the positive ion mode. Asterisks denote fragment ions originating from MNa^+ at m/z 530.
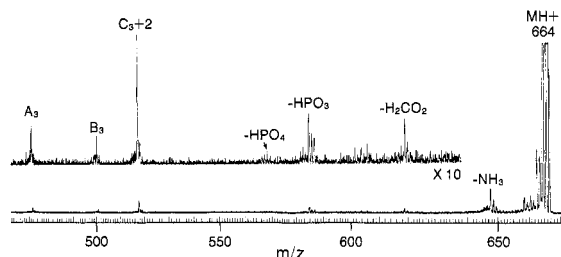

Figure 3. Metastable decomposition scan (constant B/E) of the protonated molecular ion for Arg-Tyr(PO_3H_2)-Val-Phe.

the negative ion LSIMS spectra were also less intense than those in the corresponding positive ion spectrum. The only significant peaks observed were at m/z 407 (Y_2) and 391 ($Z_2 - 1$), corresponding to loss of valine from the N-terminus, and m/z 358 (C_2), the N-terminal deprotonated amide ion resulting from loss of phenylalanine.

Because the losses of HPO_3 (–80 mu) and HPO_4 (–96 mu) were unique features of the positive and negative LSIMS spectra of both phosphotyrosine peptides, we investigated these losses further by means of a B/E = constant linked scan.[24] In the positive ion B/E linked scan of Arg-Tyr(PO_3H_2)-Val-Phe (Figure 3), small peaks were seen for losses of both HPO_3 (–80 mu) and HPO_4 (–96 mu). However, the relative abundance of the HPO_3 over HPO_4 loss was increased from about equal abundance to a fivefold excess. The increased abundance of the loss of HPO_3 over HPO_4 in the linked scans was also found in both the positive and negative ion modes for Val-Tyr(PO_3H_2)-Phe.

To determine if the phosphotyrosine peptides underwent fragmentation of the phosphate group in a manner different than peptides containing phosphoserine or sulfated tyrosine, two sulfated tyrosine-containing peptides (caerulein and CCK-8) and a phosphoserine-containing peptide (kemptide) were additionally analyzed by LSIMS. The molecular ion regions of these peptides are shown in Figures 4–6, in both the positve and negative ion modes. Almost no loss of phosphate ($-HPO_3$ or $-HPO_4$) was observed in the spectra of kemptide (Figure 4ab) while significant losses corresponding to SO_3 (–80 mu) and SO_4 (–96 mu) were observed in both the positive and negative ion LSIMS spectra of CCK-8 and caerulein (Figure 5 and 6). In particular the negative ion LSIMS spectra showed losses of SO_3 and SO_4 from the $(M - H)^-$ ions of 5–10%, similar to the losses of HPO_3 and HPO_4 found for the two phosphotyrosine peptides (see Figure 1 and 2). However, in the positive ion mode the $MH^+ - SO_3$ ions were the

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(23) The nomenclature of peptide fragments used in this paper is that of Roepstorff [Roepstorff, R.; Fohlman, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601].

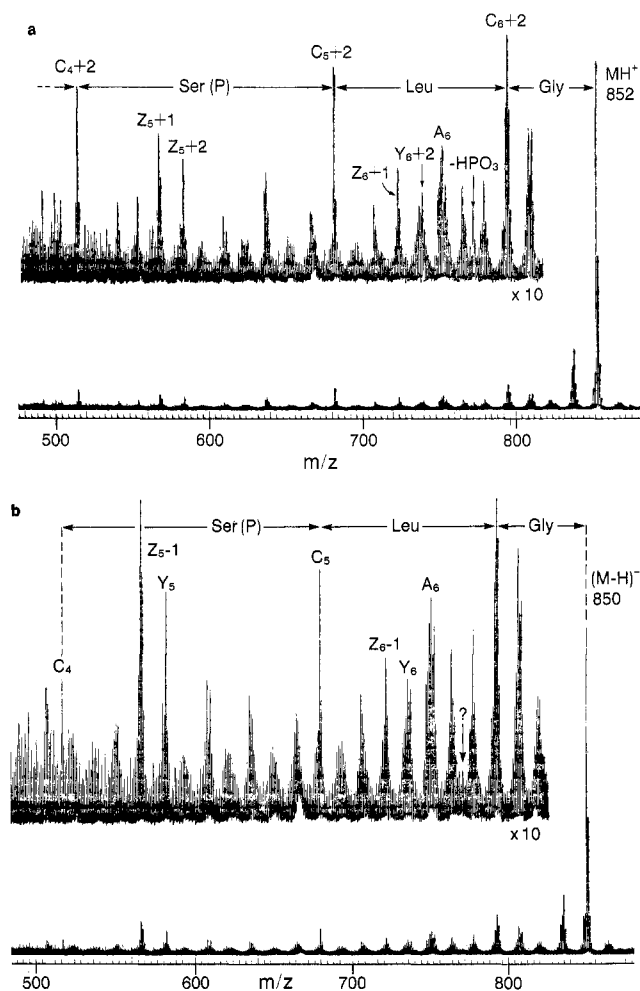(24) Boyd, R. K. *Spectros. Int. J.* **1982**, *1*, 169–200.

Figure 4. Partial LSIMS spectra of kemptide (Leu-Arg-Arg-Ala-Ser-(PO_3H_2)-Leu-Gly) in the (a) positive ion mode and (b) negative ion mode.
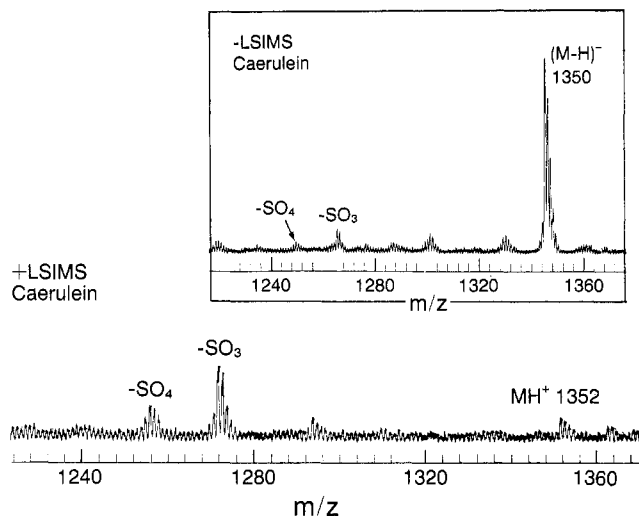

Figure 5. LSIMS spectra of the molecular ion region of caerulein (pGlu-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2) in the positive and negative (inset) ion modes.

base peaks and only weak MH^+ ions were observed.

Discussion

The identification of phosphotyrosine residues in peptides and proteins is becoming increasingly important, particularly as more growth factors and oncogene products are being identified as possessing tyrosine kinase activity.[1] The multiplicity of phosphorylation sites can give rise to a formidable analytical task,
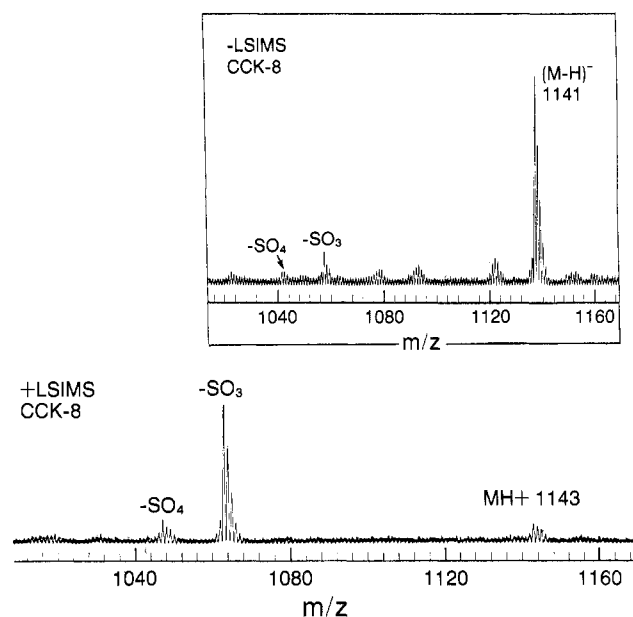

Figure 6. LSIMS spectra of the molecular ion region of CCK-8 (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) in the positive and negative (inset) ion modes.

complicated by the fact that serine and threonine residues may also be phosphorylated as has been found for the EGF receptor.[25] Current techniques for the identification and sequence analysis of phosphotyrosine peptides that involve ^{32}P labeling can be ambiguous if multiple phosphorylation sites are present.[10] The application of mass spectrometry for the direct analysis of phosphopeptides has not been extensively investigated, but from this study it is apparent that LSIMS can become an important part of this methodology.

The losses of HPO_3 and HPO_4 from the MH^+ and $(M - H)^-$ ions were the most distinctive features in all spectra obtained in these analyses. It is likely that the losses will be a common feature of the LSIMS mass spectra of most phosphotyrosine-containing peptides since the magnitudes of the HPO_3 and HPO_4 losses were essentially the same for both peptides and were present in both the positive and negative ion modes, although they were more abundant in the positive ion mode. In contrast, it has been our experience that peptides containing phosphoserine do not display any appreciable losses of this type.[26,27] For example, in the LSIMS spectrum of kemptide, Leu-Arg-Arg-Ala-Ser(PO_3H_2)-Leu-Gly, only very small ions were present above background that would correspond to loss of HPO_3 and no ions were observed for loss of HPO_4. In addition, a number of phosphoserine-containing peptides isolated from glycogen synthase were also shown to lack this fragmentation.[27] The LSIMS spectrum of caerulein[28] has been reported to show an abundant $[MH - SO_3]^+$ ion in the positive ion mode and an abundant $(M - SO_3 - H)^-$ ion in the negative mode. In these spectra, no $(MH)^+$ ion was observed at all in the positive mode, while in the negative ion mode the $(M - SO_3 - H)^-$ ion was reported to be of equal abundance to the $(M - H)^-$ ion. Our own results in the positive ion modes on caerulein and CCK-8 showed small but easily detectable MH^+ ions for both peptides with the $(MH - SO_3)^+$ peak approximately 5–10-fold larger. In the negative ion mode, the $(M - SO_3 - H)^-$ ions were not nearly as abundant as in the earlier report, suggesting that there may have been some contamination by the non-sulfated

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analogue.[29] Indeed, with purified samples we found the (M – H)⁻ ion to be about 10 times larger than the (M – SO₃ – H)⁻ ion, a ratio similar to that of the two phosphotyrosine peptides for the analogous loss of HPO₃. However, the phosphotyrosine peptides showed much more abundant molecular ions in the positive ion mode relative to the loss of phosphate than either CCK-8 or caerulein showed relative to loss of SO₃ or SO₄.

Thus, it appears from this preliminary study that phosphotyrosine-containing peptides are intermediate between the facile fragmentation of peptides containing sulfated tyrosines in the positive ion mode and the low abundance of similar fragments when phosphate is attached to serine (and probably threonine as well). While this is in agreement with one's expectations based on chemical stabilities in solution, it will require further analyses

on a more diverse class of peptides before any conclusive statements can be made about the relative ease of fragmentation of these types of peptides during mass spectrometric analysis. Nonetheless, the mass spectrometric studies suggest that the differences in fragmentation among phosphotyrosine, phosphoserine, and sulfated tyrosine-containing peptides should be of considerable analytical use and could serve as a basis for their mass spectrometric identification, particularly when both positive and negative ion LSIMS spectra are available. In more general terms, we would also expect other related ionization techniques such as fast atom bombardment or plasma desorption to be well suited for the identification and characterization of phosphorylated (and sulfated) peptides, thus increasing the applicability of the methods described in this paper.

(29) The purity of caerulein and CCK-8 was at least 99% as judged by HPLC and LSIMS analysis. No impurities were detected for the HPLC purified phosphotyrosine peptides after the purification by HPLC.

Structures of the Lithium Salts of Aromatic Secondary Amines in Weakly Polar Aprotic Solvents[1]

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Abstract: Lithium indolide (lithioindoline) in THF is shown, by vapor pressure barometry, to be a dimer, and its temperature-dependent, concentration-independent ¹³C chemical shifts are interpreted in terms of the equilibrium Li₂A₂(THF)₂ ⇌ Li₂A₂(THF)₄ (A = amide anion). Lithium 1,2,3,4-tetrahydroquinolide exhibits the same behavior. Lithium 2-methylindolide, 2-methyl-1,2,3,4-tetrahydroquinolide, and N-methyl-, N-n-butyl-, and N-isopropylanilides form the monomer LiA(THF)₃ as well as the two dimers. Monomer–dimer exchange is slow on the ¹³C NMR time scale at low (<–80 °C) temperatures. Lithium N-(2-methoxyethyl)anilide forms only Li₂A₂(THF)₂. Lithium N-tert-butylanilide exists as LiA(THF)₂ and LiA(THF)₃ depending on temperature. All the above salts form only Li₂A₂(Et₂O)₂ in diethyl ether. Both the indolide and N-tert-butylanilide salts exhibit slow E–Z isomerism on the ¹³C NMR time scale at –110 °C. ⁶Li, ¹⁵N spin–spin couplings of 3.8 and 7.5 Hz are observed for Li₂(PhNCH₃)₂(Et₂O)₂ and Li(PhNPrⁱ)(THF)₃, respectively, in the corresponding solutions below –80 °C. Rotation of the phenyl group in N-methyl-, N-n-butyl-, N-(2-methoxyethyl)-, and N-isopropyl- but not N-tert-butylanilides is slow on the ¹³C NMR time scale below –50 °C. Rotation of the tert-butyl group in LiA(THF)₃ (A = N-tert-butylanilide) is comparable with the ¹³C NMR time scale at –100 °C.

Lithium enamides (1-azaallyllithium and its derivatives) are frequently employed in synthesis as alternatives to enolate ions since the low acidities of the corresponding imines usually preclude proton transfer between reagents and products, thus eliminating further reaction (e.g., dialkylation) with the electrophilic reagent.[2] Enamide anions, like enolate ions, are ambident and do, in fact, undergo reactions at both their C and N termini.[3] There is now abundant evidence that lithium enolates exist as aggregates[4] in weakly polar aprotic solvents and that aggregation plays an important role in determining the regioselectivities of their reactions

with electrophiles.[5] It is, therefore, important to know if lithium enamides also form aggregates which similarly control their regiochemistry. In addition, strong interactions between anions and the lithium cation are evidently implicated in asymmetric syntheses which utilize the anions derived from chiral imines,[6] hydrazones,[7] oxazolines,[8] and imino ethers.[9] As part of a survey of the solution

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