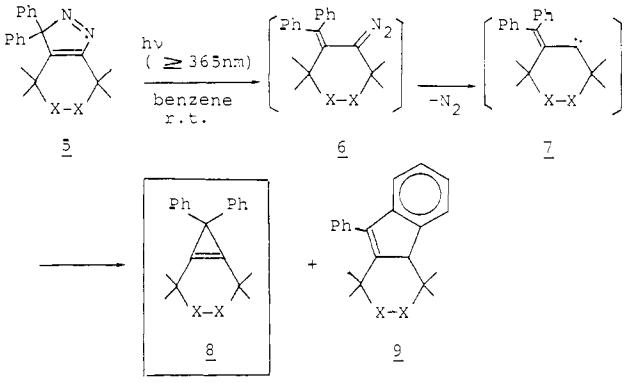
resulted in the quantitative release of nitrogen and gave a 63% yield of the fused cyclopropene 8[15] in addition to a 37% yield of the indene 9.[16,17]



The desired product **8** was separated by recrystallization from hexane–benzene at room temperature. Surprisingly, the fused cyclopropene **8** is a stable colorless crystalline compound (mp 143.5–144.0 °C). Weak absorption, presumably due to C–C double bond of the cyclopropene ring, appeared at 1805 cm^{-1} (shoulder).[15] The structure of **8** was determined by X-ray crystal structure analysis (Figure 1).[18]

Inspection of the X-ray crystal analysis data revealed the nonplanar structure of **8**, and the angle between the two rings was 162.4°, which is 7.4° larger than that estimated for **1c**.[3a,19] The bond angles of C_3–C_2–C_4 and C_2–C_3–C_5 (129.2° and 130.2°, respectively) are ca. 20° smaller than those in nonfused cyclopropene (149.9°),[20] as expected. The ^1H NMR spectrum[11] of **5** shows resonances at 1.75 (s, $CH_3 \times 4$), 3.03 (s, NCH_3), and 7.24 ppm (s, Ph) characteristic of its C_2 symmetry. The ^{13}C NMR spectrum indicates that the olefinic carbon resonance appears at 155.5 ppm.[15] Surprisingly, this value is ca. 30 ppm lower than those found for tetramethyl- (118.9 ppm) and 3,3-dimethylcyclopropenes (125.0 ppm) etc.[21] This large downfield shift should be due to the highly strained framework of **8** by ring fusion, as found in the case of the thiirene sulfoxide **3**.[11a]

The stability of **8** is clearly dependent on the α-subtitution of tetramethyl groups, which might fix the bicyclic ring system. Above all, the nonplanar structure of bicyclo[4.1.0]alkene suggested by the calculations is confirmed by the present result.

Supplementary Material Available: Listings of atomic positional and thermal parameters, bond lengths, and bond angles for compound **8** (18 pages). Ordering information is given on any current masthead page.

(13) Compound **3** reacted with ethyl diazoacetate to afford 2*H*-pyrazole, via an additional 1,3-H shift, as reported in the reaction of diphenyl thiirene sulfoxide with phenyldiazomethane: Carpino, L. A.; Chen, H.-W. *J. Am. Chem. Soc.* **1979**, *101*, 390.

(14) A methanol solution of phenanthrene (5 g/L) was used as a filtered solution (path length 1 cm).

(15) **8**: colorless crystals, mp 143.5–144.0 °C from hexane–benzene; ^1H NMR δ ($CDCl_3$) 7.24 (brs, 10 H), 3.03 (s, 3 H), 1.75 (s, 12 H); ^{13}C NMR δ ($CDCl_3$) 153.0, 144.2, 135.4, 128.2, 128.0 126.6, 63.3, 58.4, 24.6, 24.3; IR (cm^{-1}, KBr) 1805 ($\nu_{c=c}$); MS, *m/e* 387 (M^+). Anal. Calcd for $C_{25}H_{25}N_3O_2$: C, 74.39; H, 6.50; N, 10.84. Found: C, 74.31; H, 6.44; N, 10.92.

(16) **9**: oil, ^1H NMR δ ($CDCl_3$) 7.28–7.66 (s, 9 H) , 3.98 (s, 1 H), 3.06 (s, 3 H), 1.99 (s, 3 H), 1.93 (s, 3 H), 1.28 (s, 3 H), 1.09 (s, 3 H); ^{13}C NMR δ ($CDCl_3$) 155.3, 152.7, 148.4, 145.9, 139.9, 138.7, 134.9, 129.7, 128.6, 127.8, 127.7, 125.3, 123.5, 120.8, 77.3, 65.3, 62.7, 56.2, 28.1, 24.9, 21.2, 21.0; MS, *m/e* 387 (M^+).

(17) The formation of **8** and **9** is explained by the common intermediate, vinyl carbene **7**, derived from the diazo compound **6**. During the photoirradiation the reaction solution initially became light red, which corresponded to **6**, and then N_2 gas was evolved. Indene **9** may be produced from **8** via the known photoequilibrium between **7** and **8**: Halton, B.; Kulig, M.; Battiste, M. A.; Perreten, J.; Gibson, D. M.; Griffin, G. W. *J. Am. Chem. Soc.* **1971**, *93*, 2327.

(18) The crystal has monoclinic space group $p2_1/c$ with a = 10.381 (2) Å, b = 8.719 (1) Å, c = 23.717 (5) Å, and β = 98.30 (2)° with Z = 4. Intensity data were collected on a four circle diffractometer with graphite monochromated Cu Kα radiation ($3° < \theta < 120°$). 3712 unique reflections measured of which 2670 had intensities greater than $3\sigma |F_0|$ and were used for structure analysis. The structure was refined to a value of 0.086. For the detailed crystallographic data, supplementary material is available.

(19) Unfortunately, the angle for **1d** is not calculated,[3a] but is considered to be slightly larger than 155° given for **1c**.[3a]

(20) Kasai, P. H.; Meyers, R. J.; Eggers, D. F., Jr.; Wiberg, K. B. *J. Chem. Phys.* **1959**, *30*, 512.

(21) Bachbuch, M.; Grishin, Y. K.; Formanovskii, A. A. *Dokl. Acad. Nauk SSSR*, **1978**, *243*, 1171.

Fourier Transform Infrared Photoacoustic Spectroscopy: A Novel Conformational Probe. Demonstration of α-Helical Conformation of Poly(γ-benzyl glutamate)

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Fourier transform infrared photoacoustic spectroscopy (FT-IR PAS) has emerged as a novel technique for studying a wide range of problems in chemistry and biology.[1] The determination of secondary structures of biopolymers from observed vibrational frequencies is one of the long-range goals of molecular spectroscopy.[2] Biopolymers, in general, have been difficult to be investigated by conventional infrared spectroscopy due to difficulties in uniformly dispersing them into an alkali halide matrix. Incorporation into alkali halide matrix may result in structural alterations during the pelleting process[3] and hydration of the sample as well. FT-IR PAS offers an alternative method to investigate biopolymers per se in less than milligram quantities by totally eliminating artifactual effects introduced by incorporation into alkali halide matrix. FT-IR PAS represents a major advance in infrared spectroscopy which has not been extensively utilized in chemistry and biology. In this communication, the first report in the literature of an application of FT-IR PAS to the determination of molecular conformations, we are presenting the results of the application of this novel conformational probe to poly(γ-benzyl glutamate). Poly(γ-benzyl glutamate) has been shown to prefer α-helical structure in its higher molecular weight fractions by X-ray diffraction,[4] conventional IR,[5] and Raman[6] spectroscopic methods. Abe and Krimm[7] and Nevskaya and

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(1) (a) Vidrine, D. W. *Appl. Spectrosc.* **1980**, *34*, 314. (b) Krishnan, K. *Ibid.* **1981**, *35*, 549. (c) Kinney, J. B.; Staley, R. H.; Reichel, C. L.; Wrighton, M. S. *J. Am. Chem. Soc.* **1981**, *103*, 4273. (d) MaClelland, J. F. *Anal. Chem.* **1983**, *55*, 89A. (e) Rockley, M. G.; Davies, D. M.; Richardson, H. H. *Science (Washington, D.C.)* **1980**, *210*, 918.

(2) (a) Lord, R. C. *Appl. Spectrosc.* **1977**, *31*, 187. (b) Tu, A. T. "Raman Spectroscopy in Biology: Principles and Applications"; Wiley: New York, 1982. (c) Krimm, S. *Biopolymers* **1983**, *22*, 217.

(3) (a) Baker, A. W. *J. Phys. Chem.* **1957**, *61*, 450. (b) Milkey, R. G. *Anal. Chem.* **1958**, *30*, 1931. (c) for structural alterations of alkali halide matrix at high pressures, see: Knittle, E.; Jeanloz, R. *Science (Washington, D.C.)* **1984**, *223*, 53.

(4) Elliot, A.; Fraser, R. D. B.; McRae, T. P. *J. Mol. Biol.* **1965**, *11*, 821.

(5) (a) Miyawzawa, T.; Blout, E. R. *J. Am. Chem. Soc.* **1961**, *83*, 712. (b) Tsuboi, M. *J. Polym. Sci.* **1962**, *59*, 139. (c) Tomita, K.; Rich, A.; de Loze, C.; Blout, E. R. *J. Mol. Biol.* **1962**, *4*, 83. (d) Masuda, Y.; Miyazawa, T. *Makromol. Chem.* **1967**, *103*, 261.

(6) (a) Koenig, J. L.; Sutton, P. L. *Biopolymers* **1971**, *10*, 89. (b) Chen, M. C.; Lord, R. C. *J. Am. Chem. Soc.* **1974**, *96*, 4750. (c) Fasman, G. D.; Itoh, K.; Liu, C. S.; Lord, R. C. *Biopolymers* **1978**, *17*, 1729.

0002-7863/84/1506-2217$01.50/0

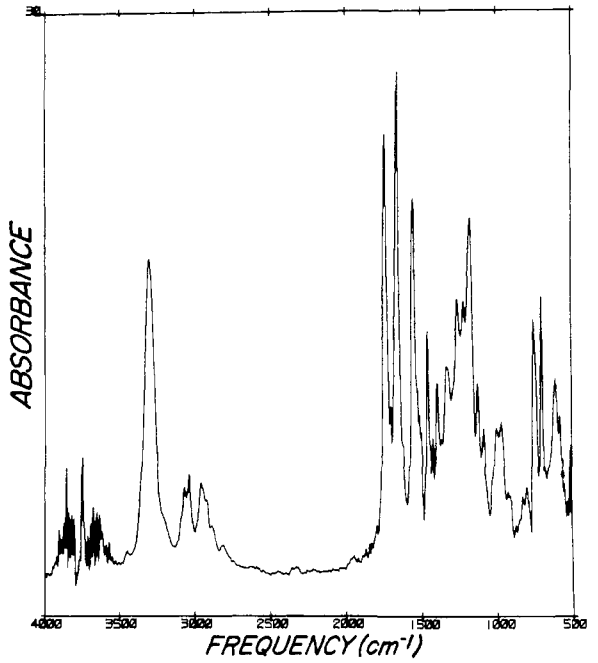

Figure 1. Fourier transform infrared photoacoustic spectrum of poly(γ-benzyl glutamate) per se, M_r 28 000 and degree of polymerization of 130, at room temperature. The spectrum shown represents 512 scans; 0.25 mg of the sample was used to obtain the spectrum.

Table I. Frequency[a] of Major Bands of Poly(γ-benzyl glutamate)

		E_1 species mode[b]		
FT-IR PAS	FT-IR poly(γ-benzyl glutamate) in KBr pellet	mean value from previous IR studies	calcd	assignment
3300	3295			amide A
1737	1736			ester carbonyl stretch
1656	1650	1654	1657	amide I
1549	1548	1548	1544	amide II
1267	1256			amide III

[a] In cm^{-1}. [b] Nevskaya and Chirgadze.[8]

Chirgadze[8] have discussed the normal vibrations of an α-helix and calculated frequencies of amide I, II, and III vibrational modes.

Poly(γ-benzyl glutamate) was commercially purchased from Sigma Chemical Co., St. Louis, MO (Lot 92F-5046), and was reported to have a molecular weight of 28 000 by viscosity measurements with a degree of polymerization of 130. No further purification was considered necessary. The basic principle of FT-IR PAS has been discussed by Krishnan.[1b] The photoacoustic signal generated when the radiant infrared light strikes the sample is picked up by a microphone and then further amplified and processed by the FT-IR spectrometer. The resulting photoacoustic interferogram is subjected to fast Fourier transform and displayed as a spectrum. The FT-IR PA spectrum of poly(γ-benzyl glutamate) per se is presented in Figure 1, and for comparison a FT-IR spectrum of poly(γ-benzyl glutamate) in KBr pellet is shown in Figure 2. The characteristics of major bands in Figure 1 are listed in Table I and compared with the frequencies observed in the polypeptide–KBr pellet spectrum, Figure 2, and with conventional IR frequencies reported in the literature.[5,8] The FT-IR PA spectrum was recorded on a Digilab FTS 20E FT-IR spectrometer at room temperature and represents the result of 512 scans. It was recorded at a resolution of 8 cm^{-1} by using a Digilab PA detector. The moving mirror in the Michelson interferometer was translated with a velocity of 0.16 cm/s, leading to modulation frequencies in the range 400–4000 cm^{-1}. Further experimental details can be found in ref 1b and in the figure legend. Polypeptide–KBr pellet spectrum was recorded on a Nicolet 7199A FT-IR spectrometer at a resolution of 4 cm^{-1}.

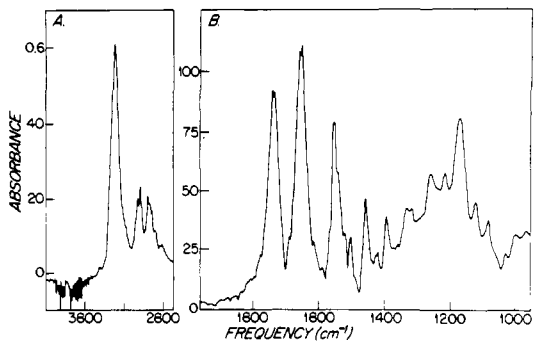

Figure 2. Fourier transform infrared spectrum of poly(γ-benzyl glutamate) in KBr pellet, M_r 28 000 and degree of polymerization of 130, at room temperature. Panel A shows the amide A and B regions and panel B shows the "Finger-print" region. A polypeptide concentration of less than 2% was used in obtaining the KBr pellet. The spectrum represents 400 scans.

Amide A band at 3300 cm^{-1} is a strong peak and is indicative of NH···O hydrogen bonding in poly(γ-benzyl glutamate). Nakamoto et al.[9] observed a correlation between NH stretching frequency and N···O bond length where N and O are hydrogen bonded. According to this observation, amide A frequency of 3300 cm^{-1} corresponds to a N···O length of ~2.80 Å. α-Helix is one of the stable conformations of the polypeptide chain, deriving its stability from NH···O hydrogen bonds. Therefore numerous studies have been directed toward an understanding of the nature of hydrogen bonds stabilizing the α-helix. Ramachandran et al.[10] found, from theoretical calculations, a N···O bond length of 2.85 Å and hydrogen bond angle, θ, of 10°, contributed to the stabilizing energy of the right-handed α-helical structure of poly(L-alanine), and it is interesting to observe the N···O distance of ~2.80 Å is in accord with the later study. It is probable that the benzyl side chain in poly(γ-benzyl glutamate) contributes to the tightening of the α-helix by stronger hydrogen bonding. Amide B band at 3035 cm^{-1} is a low-intensity peak. The CH stretching region contains a doublet with two low intensity peaks at 2954 and 2814 cm^{-1}, respectively.

The intense peak at 1737 cm^{-1} arises from the ester carbonyl stretching, which differs by ~3 to 7 cm^{-1} from values previously reported in the literature.[5] Amide I band at 1656 cm^{-1} occurs as a very strong band. Amide I frequencies, 1655 ± 3 cm^{-1}, are usually characteristic of α-helical structures,[11] the frequency depending on the strength of intramolecular hydrogen bonding. Previous studies on poly(γ-benzyl glutamate) have predicted amide I frequencies between 1650 and 1655 cm^{-1}, depending on whether it is an A or E_1 species mode, Nevskaya and Chirgadze.[8] Amide I frequency observed in this study is also quite close to a Raman frequency of 1650 cm^{-1} [12] and earlier observed by Koenig and Sutton[6a] and Chen and Lord.[6b] FT-IR PA frequencies of intense bands, viz., amide I and II bands, are usually observed to be slightly high-frequency shifted but are in reasonable accord with conventional infrared frequencies; see Table I. It was observed by Krishnan[1b] in polyethylene terephthalate that the strongest carbonyl band saturates and the weaker bands gain in intensity, sometimes leading to different band maxima in FT-IR PA spectra. Amide II band at 1549 cm^{-1} is again in the range, 1545–1549 cm^{-1}, observed for the infrared-active E_1 species mode; see Nevskaya and Chirgadze.[8] Amide III band at 1267 cm^{-1} occurs

(7) Abe, Y.; Krimm, S. *Biopolymers* **1972**, *11*, 1841.
(8) Nevskaya, N. A.; Chirgadze, Yu. N. *Biopolymers* **1976**, *15*, 637.
(9) Nakamoto, K.; Margoshes, M.; Rundle, R. E. *J. Am. Chem. Soc.* **1955**, *77*, 6480.
(10) Ramachandran, G. N.; Chandrasekharan, C.; Chidambaram, R. *Proc. Indian Acad. Sci.* **1971**, *74*, 284.
(11) Krimm, S.; Dwivedi, A. M. *Science (Washington, D.C.)* **1982**, *216*, 407.
(12) Renugopalakrishnan, V.; Bhatnagar, R. S., manuscript in preparation.

as a moderately intense band, contrary to the observations of Masuda and Miyazawa,[5d] but differs from the Raman frequency of 1296 cm^{-1} [12] and 1294 cm^{-1} reported by Chen and Lord.[6b] Previous infrared studies have reported amide III frequencies of 1280 cm^{-1} [5d] and 1314, 1328 cm^{-1}.[5b] Nevertheless, it is in the range characteristic of α-helical structures.[2b] The peak at 1467 cm^{-1} is tentatively assigned to C–C stretchings of the B_1 species mode occurring in the phenyl ring. The A_1 species mode of C–C stretchings of the phenyl ring is not observed in a FT-IR PA spectrum. The peak at 1171 cm^{-1} can be assigned to the ester, carbonyl stretch.[5b] From Table I, it can be noticed that FT-IR PA and FT-IR polypeptide–KBr pellet frequencies agree reasonably well excepting the amide III frequency. Amide III frequency of 1267 cm^{-1} observed in FT-IR PA spectrum falls in the range generally observed for α-helical structures.[2b] FT-IR PA frequencies of poly(γ-benzyl glutamate) are unabiguously characteristic of α-helical structures. Recently, FT-IR PAS has been successfully applied to several biopolymers[13] and offers itself as a novel method for obtaining infrared spectra by completely eliminating artifactual effects of incorporation into an alkali halide matrix. Its applications to the elucidation of molecular conformation far transcends biopolymer structures and has a general applicability to any chemical system.

Acknowledgment. Our thanks to Dr. K. Krishnan and S. L. Hill of Digilab Inc., Division of Bio-Rad, Cambridge, MA, for their help in the experiments described. V. Renugopalakrishnan thanks Professor M. J. Glimcher for the support and encouragement.

Registry No. Poly(γ-benzyl glutamate) (homopolymer), 25014-27-1; poly(γ-benzyl glutamate) (SRU), 25038-53-3.

(13) Renugopalakrishnan, V.; Kloumann, P. H. B.; Keith, D. A., *Science (Washington, D.C.)*, submitted.

Sequencing of Peptides by Secondary Ion Mass Spectrometry

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The advent of fast atom bombardment mass spectrometry (FABMS), or liquid SIMS, has added a new emphasis to the analysis of peptides and proteins.[1-3] The pseudo molecular ions from peptides and small proteins with molecular weight over 9000 amu have been observed by FABMS.[4] Although molecular weight information is easily obtainable, the primary sequence of the protein is very difficult to ascertain from the FABMS data due to the low degree of fragmentation and interference peaks from the liquid matrix.[5]

If one employs static secondary ion mass spectrometry (SIMS), in which one or two monolayers of the sample are placed on a surface rather than dissolved in a liquid matrix, the spectral interference from the solvent can be eliminated.[6] For the static SIMS study of most biological molecules, low current density (10^{-9} A/cm^2) primary beam conditions must be employed in order to avoid damaging the sample. With these low beam fluxes the secondary ion flux produced from a given amount of material is often low. Consequently, for many organic molecules, analysis times are long and SIMS has not been used much for their analysis.[6]

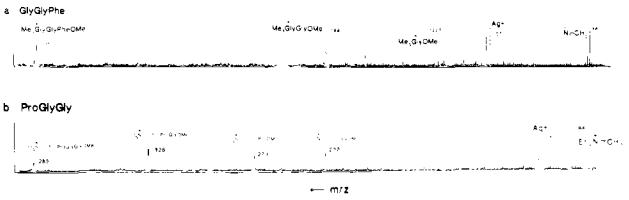
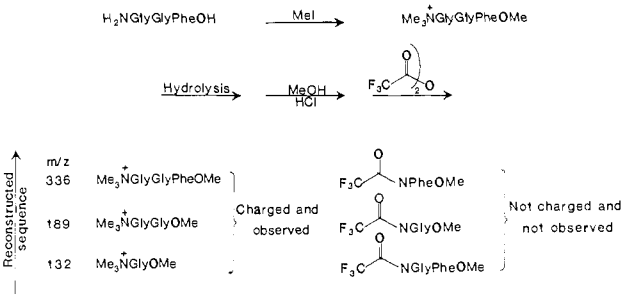


Figure 1. Static SIMS spectra of two tripeptides. (a) Glycylglycylphenyl alanine (GlyGlyPhe) quaternized with methyl iodide, cleaved, esterified, and acylated. Approximately 50 ng of each total hydrolysate were used. Beam conditions: 3 keV Ar^0 $<1 \times 10^{-9}$ A/cm^2; (b) Prolylglycylglycine (ProGlyGly) acylated with chloroacetyl chloride, cleaved, reacted with triethylamine, esterified, and acylated. Beam conditions: 3 keV Xe^0 $<1 \times 10^{-9}$ A/cm^2.

Scheme I



If the N-terminus of a peptide is labeled with a charged group and is cleaved with acid, esterified, and acylated and the SIMS spectra of the resultant mixture is obtained, then the spectra should preferentially show the ions with the charged group attached. Since these ions originate from only one end, the sequence of the peptide can be readily reconstructed on the basis of the mass differences between the ions. An outline of this sequencing method is shown in Scheme I for the tripeptide GlyGlyPhe. Figure 1 depicts the static SIMS spectra, from a silver surface, of two tripeptides labeled by two different methods at the N-terminus.

The sequence of the peptides are clearly evident from the spectra with no interferences from the uncharged materials in the matrix. (It should be emphasized that the static SIMS spectra were taken of unpurified mixtures.) The nonsequence ions at m/z 107/109, 86, and 58 in Figure 1 part a and/or b, correspond to Ag^+ ions from the substrate and amine fragment ions of the derivatizing reagents. The ion at m/z 129 in Figure 1a is attributed to an unknown impurity, but its presence causes no trouble in determining the sequence of the peptide since its mass is lower than that of the glycine derivative. Proline-containing peptides are known to form diketopiperzines.[8] If a diketopiperzine is produced, this would release free glycine, which will be derivatized and observed in Figure 1b at m/z 232.

Quaternization with methyl iodide requires severe conditions and usually gives poor yields of the quaternary ammonium de-

(1) Barber, M.; Bardoli, R. S.; Elliott, G. J.; Sedgwick, R. D.; Tyler, A. N. *Anal. Chem.* **1982**, *54*, 645A–657A.
(2) Williams, D. H.; Bradley, C.; Bojesen, G.; Sontikarn, S.; Taylor, L. C. E. *J. Am. Chem. Soc.* **1981**, *103*, 5700–5704.
(3) Hunt, D. F.; Bone, W. M.; Shabanowitz, J.; Rhodes, J.; Ballard, J. M. *Anal. Chem.* **1981**, *53*, 1704–1706.
(4) Barber, M.; Bordoli, R. S.; Elliott, G. J.; Horoch, N. J.; Green, B. N. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 753–757.
(5) Konig, W. A.; Aydin, M.; Schulze, U.; Rapp, U.; Hohn, M.; Pech, R.; Kulikhevitch, V. N. *Int. J. Mass Spectrom. Ion Phys.* **1983**, 46, 403–406.
(6) Turner, N. H.; Colton, R. J. *Anal. Chem.* **1982**, *54*, 293R–322R.
(7) Busch, K. L.; Cooks, R. G. *Science (Washington, D.C.)* **1982**, *218*, 247–254.
(8) Mazur, R. H.; Schlatter, J. M. *J. Org. Chem.* **1963**, *28*, 1025–1029. Gisin, B. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1972**, *94*, 3102–3106.