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Cysteine-linked aromatic nitriles as UV resonance Raman probes of protein structure

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Nitriles introduced into peptides and proteins can serve as useful vibrational spectroscopic probes, because the nitrile C≡N stretch is well isolated from backbone and sidechain vibrational bands. Aromatic nitriles offer large νC≡N absorption intensity in infrared spectra and resonance enhancement in Raman spectra with ultraviolet excitation. We report the ultraviolet resonance Raman spectra of cyanophenylalanine attached to cysteine, through linkage reactions that are applicable to cysteine residues in proteins. Excitation profiles are reported, and the νC≡N detection limit is estimated to be 5 μM. The band position is sensitive to solvent polarity and especially to strong H-bonding. The derivatization of mastoparan X peptide at introduced cysteine residues demonstrated the effectiveness of a cyanophenylcysteine probe in reporting the lowered environmental polarity when the peptide was incorporated into liposomes. For an asymmetrical cyanophenyl derivative, 2-CBCys, the intensity ratio of asymmetric and symmetric ring modes, $\nu 8_b$ and $\nu 8_a$, was found to respond to solvent polarity and not to H-bonding.

Supporting information can be found in the online version of this article.

Keywords: peptides; unnatural amino acids; proteins; UV resonance Raman; cyanophenylalanine

Introduction

Vibrational spectroscopy provides a powerful approach to monitor structure and dynamics in proteins because vibrational modes are sensitive to local conformation and molecular environment. However, the method is often limited by spectral crowding and the overlap of bands arising from multiple copies of the same structural elements, the peptide links[1,2] and the amino acid sidechains.[3] Introduction of additional probes, with unique vibrational signatures, can provide a valuable adjunct to protein studies. Nitrile derivatives are particularly attractive, because the νC≡N band, at ~2200 cm^{-1}, is well removed from other bands in the protein spectrum.

Because of the polarizability of the C–N bond, the νC≡N wavenumber responds to the polarity of the nitrile environment[4–7] and to the proximity of H-bond donors. Boxer and coworkers have shown that the dipolar effect is less pronounced than that of specific H-bonding and that the two effects can be distinguished by correlating νC≡N with ^{13}C nuclear magnetic resonance (NMR) chemical shifts.[8]

The νC≡N band can be measured using infrared (IR) or Raman spectroscopy. The C≡N stretch produces a strong induced IR dipole, particularly if the C≡N group is attached to an aromatic ring. The environmental sensitivity of νC≡N (Stark tuning rate) is also larger for aromatic than for aliphatic nitriles.[4,5] Nevertheless, millimolar concentrations of protein are required for IR spectroscopy. For Raman spectroscopy, however, detection limits are lowered into the micromolar range with ultraviolet (UV) excitation of the cyanophenylalanine (PheCN) Raman spectrum, thanks to resonance enhancement.

Cyanophenylalanine can be incorporated into chemically synthesized polypetides. Incorporation into recombinant proteins via the difficult method of orthogonal tRNA synthetase pairs is also possible.[9,10] Labeling of proteins by a more facile method is desirable, and the use of arylation or alkylation reactions to attach phenyl or benzyl nitriles to cysteine residues for IR studies was recently reported in a preliminary communication.[11] These methods are described more fully in the present paper, and UV resonance Raman (UVRR) characteristics of the aromatic nitrile adducts are reported.

Boxer and coworkers have incorporated the thiocyanate (−SCN) label into proteins, for IR[12] and NMR studies. To determine whether UVRR spectroscopy could be applied to these labels, we investigated the UVRR spectra of ethylthiocyanate (EtSCN). Unfortunately, resonance enhancement was found to be weak, not greater than that of cyanide ion itself, even with deep UV excitation. Thus, UVRR spectroscopy is useful only for aromatic nitriles (Fig. 1).

Methods

Raman spectra were obtained on samples sealed in 5-mm quartz NMR tubes, which were spun to ensure mixing of the solution, using 5 × 2 min of acquisition times to check for sample degradation (decrease in peak intensity *vs* $NaClO_4$ or NaCN standard and/or peak deformation) and averaged. The scattered light was collected at

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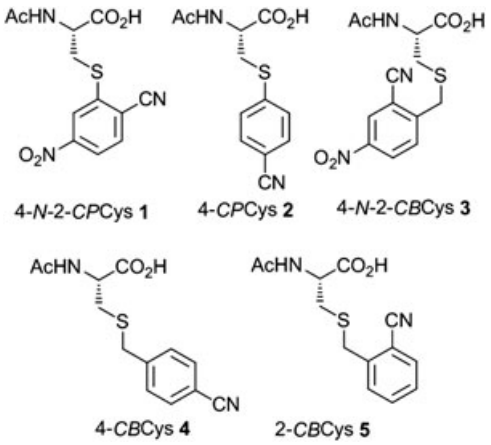


Figure 1. Aromatic nitrile derivatives of cysteine: *S*-4-nitro-2-cyanophenyl-*N*-acetylcysteine (4-*N*-2-*CP*Cys, **1**), *S*-4-cyanophenyl-*N*-acetylcysteine (4-*CP*Cys, **2**), *S*-4-nitro-2-cyanobenzyl-*N*-acetylcysteine (4-*N*-2-*CB*Cys, **3**), *S*-4-cyanobenzyl-*N*-acetylcysteine (4-*CB*Cys, **4**), *S*-2-cyanobenzyl-*N*-acetylcysteine (2-*CB*Cys, **5**).

135° geometry and focused onto a 1.26-m spectrograph (Spex 1269, 3600 grooves/mm grating) equipped with a liquid nitrogen-cooled CCD camera (Roper Scientific). The laser power at the sample was 0.5 mW. Variable wavelength excitation was provided by an intracavity frequency-doubled Ar^+ laser at 257 and 244 nm (Coherent Innova 300 FRED) and by a Q-switched frequency quadrupled Ti:sapphire laser (GM30, Photonics International, Inc.) at 197 nm (20 ns, 1 kHz) and at 212, 220 and 229 nm (0.5 μJ/pulse).[13–15]

Raman excitation profiles were determined for the $\nu C\equiv N$ bands of the cysteine derivatives in 0.1-mM aqueous solution containing 100-mM NaCN as internal standard. Raman cross-sections were calculated as previously described.[16] Aqueous stock solutions were made slightly basic (1–2 drops of 1-M NaOH added per 10 mL) to improve sample solubility and reduce the hazard of HCN production. The solvent dependence of the $\nu C\equiv N$ band was determined in H_2O, tetrahydrofuran (THF), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and cyclopentanol, using 229-nm excitation. Calibration and analysis of the spectra were carried out using GRAMS AI software (Thermo Electron Corp.). Acetone and DMSO-d_6 were used as the calibration standards.

Mastoparan X peptides were investigated at concentrations of~40 μM. DOPC (1,2-dioleoylphosphatidylcholine) vesicles (0.05 μm) were prepared by extrusion using an Avanti Polar Lipids extruder. Ascorbate was added (20x peptide concentration) to mitigate potential degradation of the CBCys or Trp residues, as had been observed for Trp and PheCN in previously investigated mastoparan X peptides.[16]

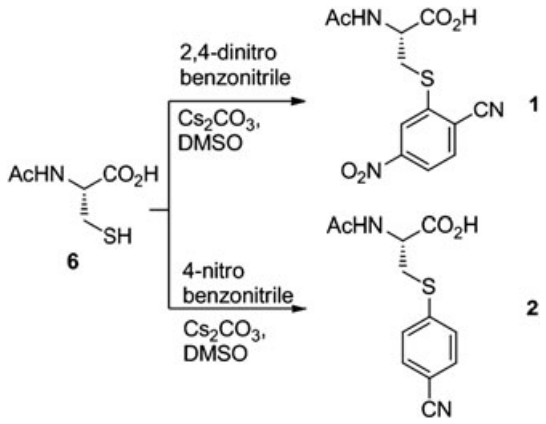
Ultraviolet–visible absorption spectra were recorded in a 0.10-cm quartz cell using an Agilent 8453 diode array spectrophotometer. Fluorescence spectra were recorded with an LS-50B Perkin Elmer Luminescence Spectrophotometer. NMR spectra were recorded using a Bruker 500-MHz instrument.

Results and discussion

Linkage chemistry

Non-aqueous preparation of 5-N-2-CPCys 1 and 4-CPCys 2

The introduction of cyanophenyl groups to *N*-acetyl cysteine **6** was achieved via nucleophilic aromatic substitution reactions using nitrobenzonitriles as electrophiles (Scheme 1).

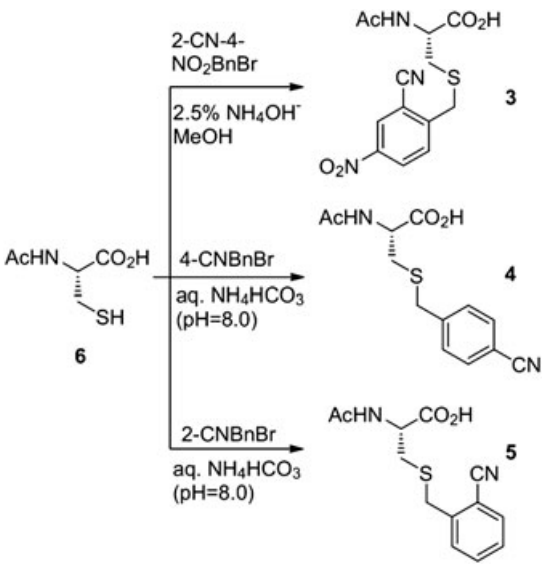


Scheme 1. *S*-arylation of *N*-acetylcysteine **6**b.

Both 2,4-dinitro benzonitrile and 4-nitro benzonitrile reacted smoothly to provide the corresponding S-arylated product 5-*N*-2-*CP*Cys **1** (5-nitro-2-cyanophenyl cysteine) and 4-*CP*Cys **2** (4-cyanophenyl cysteine), respectively, using Cs_2CO_3 as a base in DMSO.[17] The second nitro moiety in 2,4-dinitro benzonitrile was included to increase the electrophilic character of the aromatic ring but did not prove essential for the coupling reaction.

Aqueous preparation of 4-N-2-CBCys 3, 4-CBCys 4 and 2-CBCys 5

Because post-translational modification of peptides and proteins is best carried out in aqueous solution, aromatic nitrile incorporation under aqueous condition was investigated. The conventional bioconjugation techniques unique to cysteine thiols, including α-iodoacetamide or maleimide derivatives, were also considered. However, less sterically demanding benzyl bromide derivatives were selected, and the use of benzyl halide for *S*-alkylation of Cys residues[17–21] suggested that selective alkylation on the cysteine sulfur would prove viable under aqueous conditions. Indeed, the coupling of cyanobenzyl moieties to *N*-acetyl cysteine **1** was successfully achieved using either buffered NH_4HCO_3 system[20] or aqueous NH_4OH,[21] yielding 4-*N*-2-*CB*Cys **3**, (4-nitro-2-cyanobenzylcysteine), 4-*CB*Cys **4** (4-cyanobenzylcysteine) and 2-*CB*Cys **5** (2-cyanobenzylcysteine; Scheme 2)



Scheme 2. Preparation of 4-*N*-2-CBCys **3**, 4-CBCys **4** and 2-CBCys **5**.

Journal of RAMAN SPECTROSCOPY

Attachment of probes to mastoparan X

Because our goal was an accessible route to a minimally disruptive post-translational addition of probe moieties, we verified that our linkage chemistry could be used to attach the probes to whole peptides. We chose mastoparan X as our model system, as it associates with lipid membranes, allowing for hydrophobic burial of the probe, in the presence of lipids.[22] Mastoparan X was previously used in our UVRR study of the PheCN probe, which was incorporated by peptide synthesis.[16] Four consecutive residues (positions 2–5) were chosen for mutation to cysteine and subsequent alkylation with 4-cyanobenzyl bromide.

We first attempted direct solid-phase installation of this probe by alkylation, which could facilitate purification and allow for other modification. Two cysteine variants of mastoparan X (K4C and G5C) were prepared using conventional fluorenylmethyloxycarbonyl-based solid-phase peptide synthesis. Selective 4-methoxytrityl (*para-Monomethoxytrityl*) deprotection on cysteine (structure **7** – Scheme 3) was achieved by 1% trifluoroacetic acid/triisopropylsilane/dichloromethane treatment,[23] and the subsequent alkylation was smoothly accomplished via identical conditions to yield the desired peptides **8** and **9** (Scheme 3). Polyethylene glycol-based resin was used because of its convenient swelling in aqueous buffer, but polyethylene glycol-based resins are not strictly required for probe incorporation.[24]

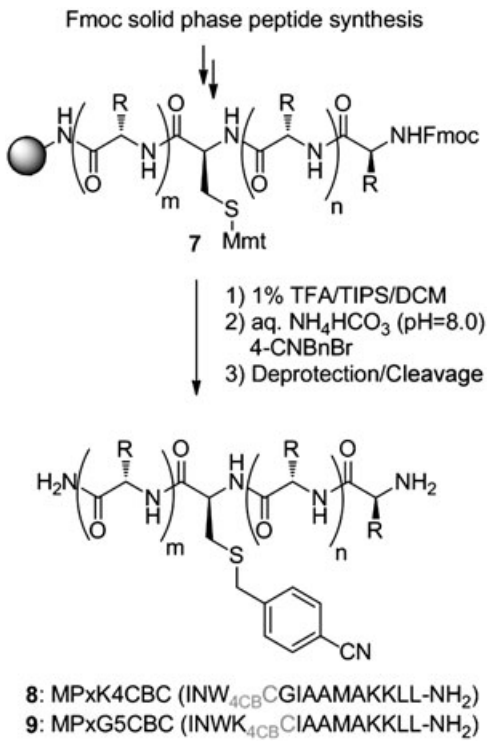
Next, we turned our attention to the solution-phase method that could be useful for probe installation in native peptides and proteins. The other two cysteine variants of mastoparan X (N2C and W3C) were prepared via standard fluorenylmethyloxycarbonyl solid-phase peptide synthesis. After cleavage and deprotection, the purified peptides were subject to conditions identical to standard *N*-acetyl cysteine alkylation, again using 4-cyano benzyl bromide to introduce the probe to the cysteine residue (Scheme 4). It is noteworthy that the reaction was very selective and high yielding as shown in the high-performance liquid chromatography profile of the crude alkylation reaction mixture. (See supporting information, Part 1).

Characterization

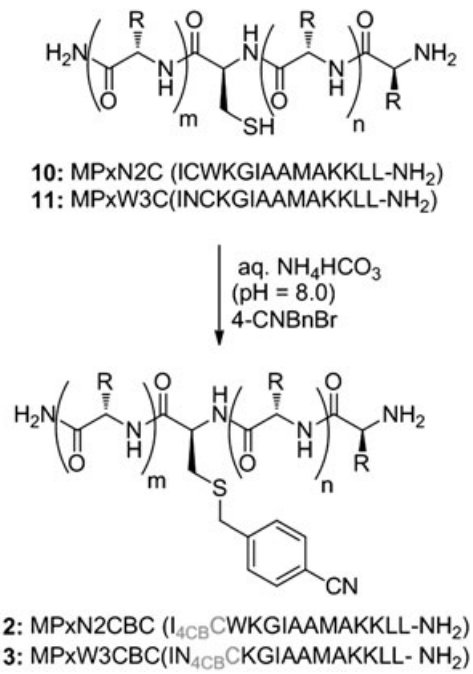
Stability

The stability of the cysteine derivatives was monitored, as photodegradation can be a problem when UV excitation is used to acquire Raman spectra. Considerable photodegradation was evident for an aqueous solution of 5-N-2-CPCys after acquisition of consecutive Raman spectra. The position of the $\nu C\equiv N$ band shifted from 2230 to 2242 cm^{-1}, and the peak broadened and weakened. (Figs. S3–S5, supporting information). 4-N-2-CBCys also showed poor stability in aqueous solution, even in the absence of laser exposure. After a sample was stored overnight (in the dark at 4 °C), the intensity of the $\nu C\equiv N$ band had decreased by ~15%, and there was a 4-cm^{-1} shift in its position. (Fig. S6, supporting information). Degradation of both derivatives was evidenced by a color change (from orange to pale yellow) and the appearance of new species in the NMR spectrum (Fig. S5 right panel, supporting information). The similar chemical shifts and coupling constants for all but the sidechain methylene (beta) protons suggests that the initial degradation event is cleavage at the S–C bond. However, after >3 h of laser exposure, a complex mix of products is evident (Fig. S5 last panel, supporting information).

The instability is attributable to the electronic effects of the nitro substituents, because none of the compounds lacking the nitro groups, 2-CBCys, 4-CBCys or CPCys, showed any change in the $\nu C\equiv N$ position or other signs of photodegradation during Raman spectral acquisition. A significant decrease in the band intensity (>10%) was only observed after storage of the solutions for extended periods (10+ days; Figs. S7–S9, supporting information).



Scheme 3. On resin probe incorporation.



Scheme 4. Solution-phase probe incorporation.

Fluorescence

Although 4-PheCN fluoresces strongly,[25,26] and has been used in fluorescence energy transfer (FRET) measurements with tryptophan residues as acceptors,[25,27] none of the cysteine-linked PheCN derivatives showed any appreciable fluorescence. It is likely that the fluorescence is quenched by the cysteine sulfur atom.

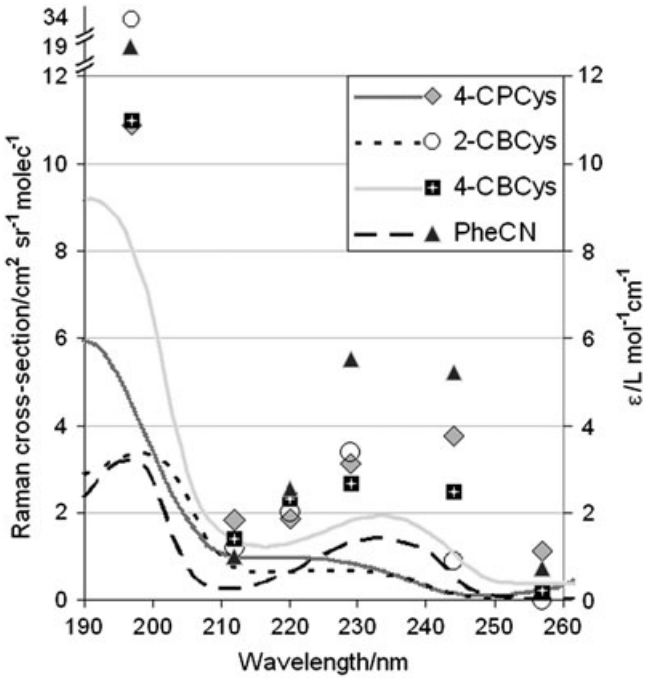


Figure 2. Raman excitation profiles (symbols) and UV–visible absorption spectra (lines) for NCPCys, NCBCys, CPCys, 2-CBCys and 4-CBCys.

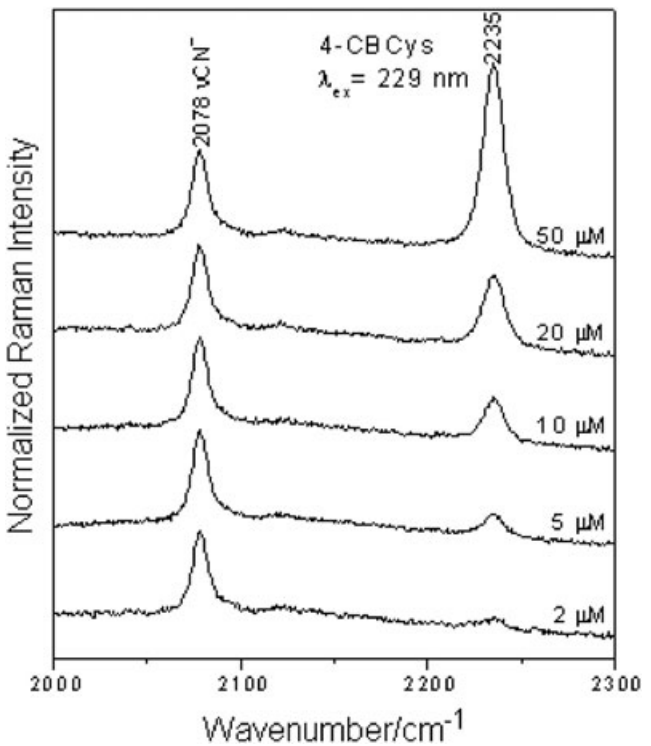


Figure 3. UV Raman spectra of 4-CBCys at the indicated concentrations, showing its $\nu C \equiv N$ band along with that of the CN^- from the 100-mM NaCN intensity standard.

UV resonance Raman enhancement

The $\nu C \equiv N$ band was strongly enhanced with UV excitation for all cysteine derivatives, as it is for PheCN itself.[16] Excitation profiles (Fig. 2) show maxima near 235 nm, coincident with the absorption bands in this region, indicating resonance with the benzene-like L_a electronic transitions.[28] Significantly larger cross-sections were seen with 197 nm excitation, in resonance with the stronger benzene-like $B_{a,b}$ electronic transitions. However, the noise level at 197 nm was greater than at 229 nm, due (at least in part) to the superior efficiency of our detector at longer wavelengths. Signal/noise was optimal at 229 nm, as was found for PheCN.[16] Consequently, 229 nm excitation was used for studying detection limits and solvent effects.

Figure 3 shows the intensity variation of the 2-CBCys $\nu C \equiv N$ band as the concentration is decreased from 50 to 2 μM, in 100 mM NaCN, present as an internal standard. Although the band is detectable at 2 μM, we judge 5 μM to be the practical detection limit. A similar result was obtained for 4-CBCys.

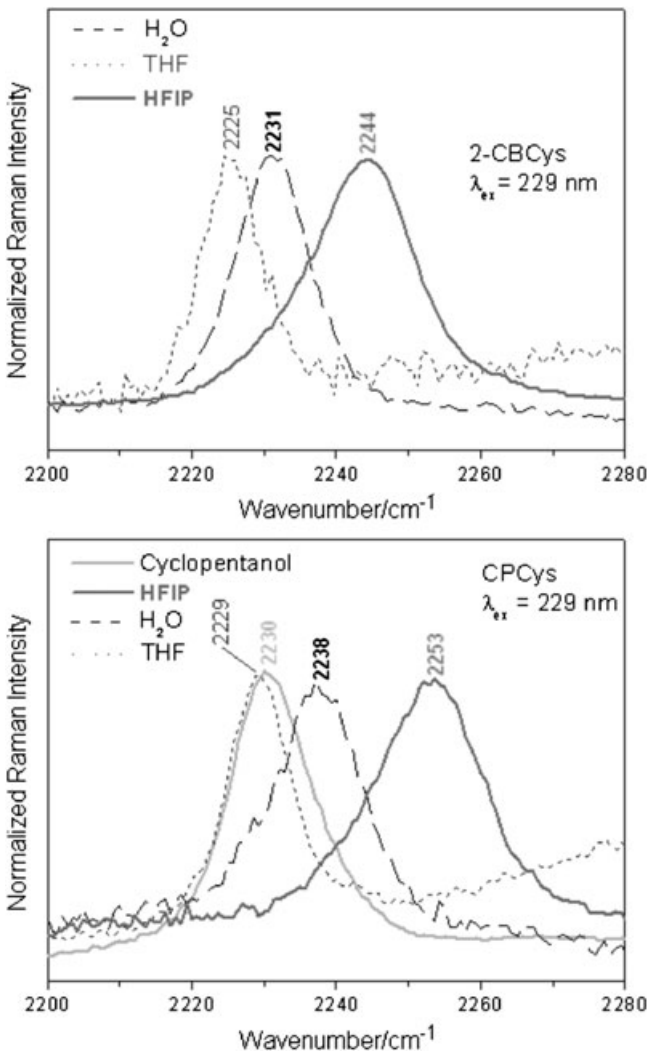


Figure 4. Solvent-dependent shifts in the $\nu C \equiv N$ bands of 4-CBCys in H_2O (200 μM), THF (500 μM) and HFIP (360 μM) and CPCys in H_2O (100 μM), THF (100 μM), HFIP (90 μM) and cyclopentanol (saturated solution). $\nu C \equiv N$ intensities normalized.

Journal of RAMAN SPECTROSCOPY

For comparison with the aromatic Cys derivatives, EtSCN (ethyl thiocyanate) was investigated with 197 nm excitation, because its absorbance peak near 197 nm suggested potential resonance enhancement for UVRR. Thiocyanate adducts of proteins are readily prepared by reacting cysteine sidechains with cyanide and have been exploited by Boxer and coworkers as IR probes. Unfortunately, the $\nu C\equiv N$ intensity of EtSCN was similar to that of free cyanide (Fig. S10, supporting information). Ala-CN was tested and also failed to display resonance enhancement beyond that of free cyanide.

Solvent and H-bonding dependence

To assess the environmental sensitivity of the $\nu C\equiv N$ band, we recorded Raman spectra of 4-CBCys, CPCys (Fig. 4) and 2-CBCys (Fig. S1, supporting information) in a variety of solvents. As seen previously for PheCN, the wavenumber depends on solvent polarity, shifting up in aqueous solution (~2236 cm^{-1}) from its value in THF or cyclopentanol (~2230 cm^{-1}). However, a much higher wavenumber, ~2250 cm^{-1}, was produced in HFIP. HFIP has a lower dielectric constant than cyclopentanol (Table 1), but it is known to form unusually strong H-bonds, an effect clearly seen in the anomalously high $\nu C\equiv N$. Boxer and coworkers have reported specific H-bond effects on $\nu C\equiv N$ of EtSCN and have shown how to distinguish them from polarity shifts, using combined IR and ^{13}C NMR measurements.[8]

Table 1. Comparison of dielectric constants for different solvents

Solvent	$\nu C\equiv N$ (cm^{-1})			$\nu 8a/b$ ratio	Dielectric constant[31]
	CPCys	4-CBCys	2-CBCys	2-CBCys	
H_2O	2238	2235	2231	2.50	80.10[a]
Cyclopentanol	2230	(No data)	(No data)	(No data)	18.5[c]
HFIP	2253	2250	2244	2.05	16.70[a]
THF	2229	2228	2225	1.35	7.52[b]

HFIP, hexafluoroisopropanol; THF, tetrahydrofuran.
[a]at 293 K;
[b]at 295 K;
[c]at 288 K.

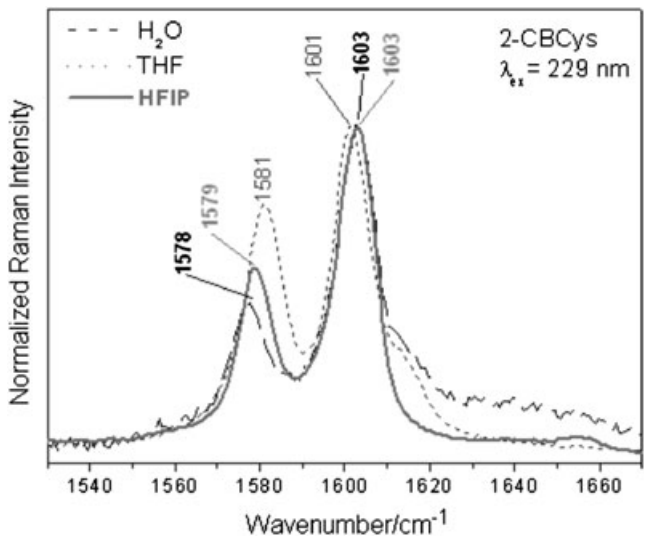


Figure 5. Solvent-dependence of the $\nu 8_a$-type and $\nu 8_b$-type bands of 2-CBCys in H_2O (200 μM), THF (saturated solution) and HFIP (450 μM).

Interestingly, we found a direct correlation with solvent polarity for the intensity ratio of the $\nu 8_a$-type and $\nu 8_b$-type aromatic ring modes of 2-CBCys (Fig. 5 and Table 1). These are stretching modes of the phenyl ring, with orientations parallel ($\nu 8_a$) and perpendicular ($\nu 8_b$) to the pseudo twofold axis, defined by the CN substituent. If this axis is maintained, as in 4-CBCys, the perpendicular mode is weak; but it gains strength when the symmetry is perturbed, as in 2-CBCys. The perturbation is seen to depend on the solvent, with the $\nu 8_b$ relative intensity increasing in the order THF < HFIP < water. The fact that HFIP is *not* an outlier in this series indicates that the perturbation results from a dipole–dipole interaction, rather than from H-bonding. Thus, the $\nu 8_b/\nu 8_a$ intensity ratio could be used in conjunction with the $\nu C\equiv N$ shift to distinguish H-bonding and polarity effects when 2-CBCys is used as a UVRR probe.

Mastoparan X peptides

The four 4-CBCys-containing mastoparan X peptides revealed 5–6 cm^{-1} downshifts between aqueous solution and DOPC liposomes (Fig. 6), consistent with transfer of the probes to a less polar environment. When bound to liposomes, the peptides form amphiphilic helices,[29] perpendicular to the lipid axis.[30] However, the observed shifts do not distinguish which side of the helix is labeled, indicating that sidechains on the solvent-facing side of the helix are shielded from solvent by the lipid head groups. A similar result was reported from a previous IR study of mastoparan X having synthetically incorporated PheCN probes.[22]

We note that the degradation observed in our previous UVRR study of mastoparan X-bearing liposomes[16] was greatly reduced by the inclusion of ascorbate, in 20 molar excess, in the buffer. In addition to its anti-oxidant properties, ascorbate acts as a UV shield for molecules in the laser path, including those at the rear of the sample tube, outside of the focal volume from which Raman scattering was collected by the optics. Interference from the ascorbate Raman spectrum was negligible.

Conclusions

Cyanophenyl groups can be linked to the thiolate group of cysteine using electrophilic aromatic substitution with nitrocyanophenyl derivatives or via alkylation of the thiolate with cyanobenzyl

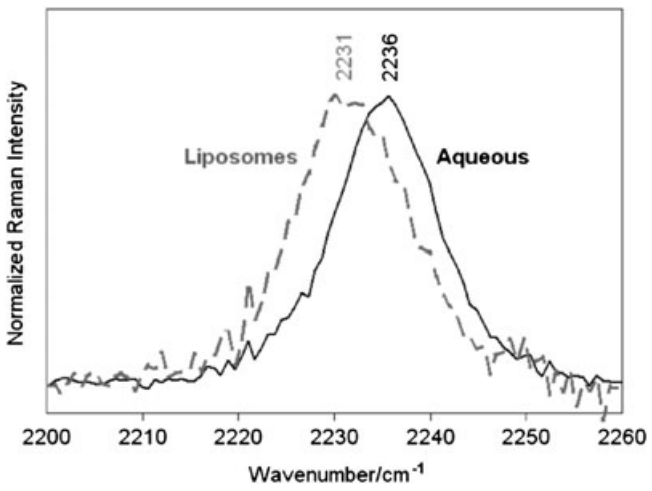


Figure 6. UV Raman spectra of mastoparan X W3CBCys derivative in aqueous buffer with (dashed line) and without (solid black line) DOPC liposomes (Concentration: ~50 μM). See figure S12 for all mastoparan X peptide data.

bromide derivatives. The latter route, employing aqueous chemistry, is more amenable to peptide and protein labeling.

The resulting cysteine-linked cyanophenyl or cyanobenzyl labels afford intense UVRR bands for the isolated $\nu C\equiv N$ mode, which serves as a monitor of the probe environment. Its wavenumber increases with increasing polarity and is particularly sensitive to strong H-bond donation. The 4-CBCys probe attached to cysteine residues introduced into mastoparan X reveals a less polar environment when the peptide inserts into liposomes. The $\nu C\equiv N$ shift is the same for probes attached to either side of the amphiphilic helix, indicating that sidechains facing the surface are also protected from solvent. PheCN and its derivatives greatly outperform their tyrosine and, especially, phenylalanine analogs as Raman probes because of both superior enhancement over a broad range of wavelengths and also the isolated, environment-sensitive $\nu C\equiv N$ band.

For the 2-CBCys molecule, the $\nu 8_b/\nu 8_a$ intensity ratio increases with solvent dielectric constant, without anomalous values associated with strong H-bonding. This probe could therefore be used to disentangle specific H-bond and general polarity effects, analogous to the combination of IR and ^{13}C NMR spectroscopy used by Boxer and coworkers for thiocyanate probes.[8]

Supporting information

Supporting information can be found in the online version of this article.

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