Nitro-heteroaromatic Derivatives of Amino-acids and Peptides. Part IV.[1] A Circular Dichroic Method for the Selective Determination of Cysteinyl Residues†

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Cysteinyl residues react with 2-fluoro-3-nitropyridine rapidly and under mild conditions. The c.d. spectra of the resulting chromophoric *S*-3-nitro-2-pyridyl derivatives have been recorded. The results suggest the application of the reaction to the analytical determination of thiol groups in peptide molecules.

WE have demonstrated [1,2] that 2-fluoro-3-nitropyridine can be successfully employed for determination of *N*-terminal amino-acids in peptides and to obtain evidence for the absolute configuration of their asymmetric α-carbon atoms. Furthermore, it appears that the 3-nitro-2-pyridylamino-chromophore is a promising u.v.–visible absorption and c.d. 'reporter group.' [1,3] It is known that halogeno-nitroaromatic compounds react rapidly with thiol groups in neutral or slightly alkaline solution,[4] giving chromophorically substituted thio-derivatives. We describe here an extension of our investigation to the spectral and chiroptical properties of *S*-2-Npy-cysteinyl derivatives and related compounds.

EXPERIMENTAL

Materials.—All chemicals used were of analytical reagent grade. Glutathione, L-cysteine, dinitrofluorobenzene, and 2-chloro-3,5-dinitropyridine were purchased from Fluka (Switzerland). *N*-Acetyl-L-cysteine was obtained from Nutritional Biochemicals Corporation. 2-Fluoro-3-nitropyridine and 2-fluoro-5-nitropyridine were synthesized as previously described.[5]

Methods.—Most of the appropriate information is given in the preceding paper. The purity of compounds was tested by t.l.c. on silica gel G in the systems (i) n-butanol–acetic acid–water (6 : 2 : 2), and (ii) chloroform–methanol–acetic acid (95 : 5 : 1). Compounds were located visually or with the aid of a u.v. lamp. The progress of the reaction between thiol groups and 2-fluoro-3-nitropyridine was followed at constant pH by the use of a Radiometer TTT1c titrator with a combination electrode GK2021.

N-*Acetyl*-S-3-*nitro*-2-*pyridyl*-L-*cysteine*.—To *N*-acetyl-L-cysteine (3·0 g, 18·4 mmole) in 5% sodium hydrogen carbonate (30 ml) 2-fluoro-3-nitropyridine (2·87 g, in 20·2 mmol) in ethanol (30 ml) was added with stirring at room temperature. After 15 min the solution was concentrated to a small volume under reduced pressure, extracted twice with ether and then acidified with 6M-hydrochloric acid (Congo Red). The solid which separated was extracted with ethyl acetate and the solution was dried (Na_2SO_4), filtered, and evaporated under reduced pressure. The solid residue was treated with light petroleum ether and crystallized from ethanol–water to give the *derivative* in quantitative yield; m.p. 156—159° (Found: C, 41·8; H, 4·0; N, 14·4; S, 10·8. $C_{10}H_{11}N_3OS_5$ requires C, 42·2; H, 3·9; N, 14·7; S, 11·2%).

N-*Acetyl*-S-5-*nitro*-2-*pyridyl*-L-*cysteine*.—This *derivative* was similarly obtained from *N*-acetyl-L-cysteine and 2-fluoro-5-nitropyridine in quantitative yield; m.p. 167—169° (Found: C, 41·8; H, 3·8; N, 14·5; S, 10·9%).

N-*Acetyl*-S-3-*nitro*-2-*pyridyl*-L-*cysteine Methyl Ester*.—A solution of 2-fluoro-3-nitropyridine (5·56 g, 40 mmol) in ethanol (50 ml) was added to *N*-acetyl-L-cysteine methyl ester (6·2 g, 38 mmol) in 5% sodium hydrogen carbonate (50 ml) with stirring at room temperature. After 20 min the yellow *precipitate* was filtered off, washed with ether and water, and crystallized from ethanol–light petroleum (1 : 3); yield 10 g (87%), m.p. 152—153° (Found: C, 44·6; H, 4·5; N, 13·9; S, 10·5. $C_{11}H_{13}N_3O_5S$ requires C, 44·2; H, 4·4; N, 14·1; S, 10·7%).

N-*Acetyl*-S-2,4-*dinitrophenyl*-L-*cysteine*.[6]—To *N*-acetyl-L-cysteine (2 g, 12·4 mmol) in 5% sodium hydrogen carbonate (30 ml) 2,4-dinitrofluorobenzene (2·27 g) in ethanol (30 ml) was added; the mixture was stirred at room temperature for 10 min, evaporated under reduced pressure, extracted twice with ether and then acidified with 6·0M-hydrochloric acid (Congo Red). The *solid* which separated was filtered off, washed with water, and crystallized from ethanol–water; yield 4 g (98%), m.p. 142—145° (Found: C, 39·8; H, 3·5; N, 12·6; S, 9·7. $C_{11}H_{11}N_3O_7S$ requires C, 40·2; H, 3·4; N, 12·8; S, 9·7%).

S-3-*Nitro*-2-*pyridylglutathione*.—To a solution of reduced glutathione (3·06 g, 0·01 mol) in 1·0M-potassium hydrogen carbonate (40 ml) was added 2-fluoro-3-nitropyridine (1·56 g, 0·011 mol) in methanol (5 ml). The mixture was stirred for 15 min and then acidified to pH 2 with dilute hydrochloric acid. The yellow *product* (3·9 g, yield 92%) was washed with ether and acetone; m.p. 159—161° (Found: C, 41·4; H, 4·7; N, 16·3; S, 7·6. $C_{15}H_{19}N_5O_8S$ requires C, 42·3; H, 4·5; N, 16·4; S, 7·5%).

RESULTS AND DISCUSSION

The thiol groups of cysteine derivatives were nitropyridylated with 2-fluoro-3-nitropyridine and 2-fluoro-5-nitropyridine, at pH 8—9 and at room temperature, in about 15 min. The derivatives were obtained in crystalline form by acidification of the reaction mixtures at pH 2—3. The progress of the reactions was controlled by measuring the uptake of alkali with a pH-stat;

† The following abbreviations are used: 3-Npy 3-nitro-2-pyridyl; 5-Npy 5-nitro-2-pyridyl; Dnpy 3,5-dinitro-2-pyridyl: Dnp 2,4-dinitrophenyl.

[1] Part III, C. Toniolo, D. Nisato, L. Biondi, and A. Signor, preceding paper.

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the pH dependence of the rate revealed that the thiol groups are readily modified in slightly alkaline solution, whereas below pH 6·0 no appreciable reaction was detected. Furthermore, it was observed that the compounds are not stable under strongly alkaline conditions. The possibility that other nucleophilic systems present in peptide molecules might compete for 2-fluoro-3-nitropyridine with thiol groups was also examined. In this respect, Wallenfels *et al.*[7] demonstrated that the reactivity of thiol groups towards fluoro-nitroaromatic compounds at pH 8·4 is *ca.* ten times higher than that of amino- and phenolic groups and *ca.* one hundred times higher than that of imidazole systems. Our findings relative to the synthesis of *S*-3-Npy-glutathione have shown that, under the aforementioned conditions, selective modification of the thiol function, relative to the α-amino-group, takes place.

The absorption spectrum of *N*-acetyl-*S*-3-Npy-L-cysteine in water in the 300—400 nm region exhibits a band near 365 nm associated with the 3-nitro-2-pyridylamino-chromophore. The absorption band of the isomeric 5-Npy-derivative associated with the 5-nitro-2-pyridylthio-chromophore is centred at 340 nm. The molar extinction coefficient at $\lambda_{max.}$ is much higher for 5-Npy- than for 3-Npy-derivatives; in analogy with 3-nitro-2-pyridylamino-compounds a considerable steric inhibition of resonance could explain the experimental data.[1] Furthermore, *S*-Dnpy-derivatives[8] and *S*-Dnp-derivatives present an intense absorption band near 340 nm with a shoulder at *ca.* 365 nm. Parallel results have been obtained for analogous compounds, *e.g.* tryptophan derivatives containing nitrophenylsulphenyl groups in the 2-position of the indole nucleus[9] and nitrophenylthioethers.[10-12]

The effect of solvent polarity on the u.v. absorption spectrum of *N*-acetyl-*S*-3-Npy-L-cysteine has been investigated and compared with that of *N*-3-Npy-derivatives; the range of wavelength maxima of the former (from 370 nm in water or dimethyl sulphoxide to 362 nm in n-hexane–dioxan) is much narrower than that of the latter (30 nm).[1] This might be explained in terms of a less effective participation of sulphur *d*-electrons in the π-electron system of the chromophore in comparison with nitrogen *p*-electrons, and hence in terms of lower polarity of the ground state of 3-nitro-2-pyridylthio-compounds. Consequently, this chromophore does not appear to be as promising an environmentally sensitive u.v.-absorption ' reporter group ' as the *N*-3-Npy chromophore.[1] Nevertheless, an encouraging result has recently been reported[13] in this area, *i.e.* that use of a difference spectroscopy technique can give information concerning the polarity of microenvironments of proteins in which *S*-nitroaromatic chromophores (and hence cysteine residues) are located.

Since the 365 nm absorption of *S*-3-Npy-compounds exhibits a rather low extinction value and since it occurs in a spectral range which is transparent in peptide molecules, we have investigated in detail the c.d. of *S*-3-Npy-cysteinyl derivatives in the 300—500 nm region. The c.d. spectrum of *N*-acetyl-*S*-3-Npy-L-cysteine in water shows a *positive* Cotton effect centred at 367 nm. The *S*-5-Npy-analogue exhibits a small *negative* Cotton effect centred in the 320—340 nm region, and the *S*-Dnp-analogue shows a single positive c.d. band near 365 nm. As in the case of the nitropyridylamino-compounds,[1] the c.d. spectra of *S*-Dnp- and *S*-Dnpy-derivatives[8] reveal the absorption band which is covered in the u.v. spectra. Furthermore, since *S*-3-Npy-derivatives show much more favourable $\Delta\varepsilon/\varepsilon$ ratios in the near u.v. region than their 5-Npy-, Dnpy-, and Dnp-analogues, the *S*-3-Npy-derivative seems by far the most useful for c.d. studies.

On changing the solvent from water or dimethyl sulphoxide to solvents of lower polarity, such as dioxan, chloroform and benzene, *N*-acetyl-*S*-3-Npy-L-cysteine methyl ester experiences only a small hysochromic shift in the c.d. wavelength maxima (*ca.* 6 nm). In addition, it is impossible to correlate the observed variations in $\Delta\varepsilon_{max.}$ with solvent polarity. However, the c.d. spectrum of papain containing a Dnp group covalently bound to cysteine in position 24 shows a large positive Cotton effect near 325 nm, which corresponds to a blue shift and an increase in intensity in comparison with model peptides.[13] In conclusion, besides the potential usefulness of *S*-3-Npy as a c.d. ' reporter group,' the sign of its Cotton effect can safely be used to determine the configuration of the asymmetric carbon atom of cysteinyl residues in peptide molecules.*

With reference to the selectivity of the reaction of 2-fluoro-3-nitropyridine with cysteinyl residues, we have investigated in detail the reactions with glutathione and cysteine by means of a c.d. technique (observation at 365 nm). In the presence of a ten-fold excess of 2-fluoro-3-nitropyridine, in aqueous 5% sodium hydrogen carbonate–ethanol (4 : 1) and at room temperature, the thiol group of glutathione reacts quantitatively in about 4 min, whereas after 10 min the 425 nm band, diagnostic of reaction with the α-amino-group,[1] is not yet detectable. These results confirm that selective modification of

* A recent aspect of stereospecificity–bioluminiscence relationships, represented by the work of McElroy *et al.*,[14] illustrates the major role of cysteine configuration in the natural products field. In addition, it has recently been demonstrated that two peptide antibiotics, malformin[15] and siomycin A,[16] contain D-cysteine residues.

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SH in the presence of NH_2 groups is readily achieved under these conditions. However the c.d. spectrum of the reaction mixture in the 300—500 nm region after 330 min shows two Cotton effects, the former small and negative at about 430 nm and the latter large and positive at 367 nm. By comparison with c.d. spectra of model compounds we assign the two bands to the optically active 3-nitro-2-pyridylamino- and 3-nitro-2-pyridylthio-chromophores, respectively. Since L-glutamic acid is the *N*-terminal amino-acid of glutathione, the 430 nm c.d. band is negative.[1] Therefore c.d. appears to be a promising technique for following the reaction of 2-fluoro-3-nitropyridine with SH and NH_2 groups in peptide molecules. In contrast, if a ten-fold excess of 2-fluoro-3-nitropyridine reacts with L-cysteine in aqueous 5% sodium hydrogen carbonate–ethanol (4 : 1) at room temperature, the positive c.d. band at 367 nm and the negative band at 425 nm increase simultaneously. This confirms that the amino-group of cysteine reacts much faster than that of glutathione.[7] This effect can be interpreted in terms of an interaction between the neighbouring SH and NH_2 groups of cysteine. It is likely that nucleophilic substitution takes place in the first stage at the sulphur atom and that, in a second stage, the aryl system migrates intramolecularly to the NH_2 group. As a result of these studies, we suggest that when thiol and amino-groups are suitably positioned relative to one another they react as a single functional group with electrophilic compounds. In the SH/NH_2 system of cysteine the reactivity of either group is influenced by the other. The situation may be described as a polar interaction or in terms of a hydrogen bridge.

We have applied the c.d. technique in the titration of the SH function in peptide molecules with 2-fluoro-3-nitropyridine[17] through examination of the 367 nm band. The absorption at 367 nm increases linearly with the [pyridine derivative]/[SH] molar ratio in the 0·1—1·0 range. Above 1·0 molar ratio, no further increase is observed, indicating the end-point of formation of the chromophoric derivative. Thus, since the reaction between 2-fluoro-3-nitropyridine and cysteinyl residues under suitable conditions is selective and quantitative, evaluation of the extent of chemical and enantiomeric purities in the synthesis of cysteine-containing peptides by means of this procedure appears to be feasible. These conclusions were supported by the observation that the concentrations used for c.d. determinations (*ca.* 0·1%) and the nature of the solvent mixtures employed safely allowed one to assume the identity of enantiomeric and optical purities. However, the point that the position and intensity of the Cotton effect under investigation are solvent-dependent must be emphasized; therefore the chiroptical properties of a model compound must initially be verified in the solvent selected for the quantitative determination.

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