Nitro-heteroaromatic Derivatives of Amino-acids and Peptides. Part III.[1] Application of Ultraviolet–Visible Absorption and Circular Dichroism to *N*-(3-Nitro-2-pyridyl)amino-acids†

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U.v.–visible absorption and c.d. curves have been recorded for *N*-(3-nitro-2-pyridyl)amino-acids and compared with those of related compounds. The chiroptical properties of these compounds, along with the chemical behaviour of 2-fluoro-3-nitropyridine, suggest a novel procedure for determining the absolute configuration of *N*-terminal amino-acids of peptides.

ULTRAVIOLET–VISIBLE and n.m.r. studies [2-4] of *NN*-dialkylamino-*ortho*-nitroaromatic compounds have established that the out-of-plane rotation necessary to relieve steric interactions is shared by the nitro- and dialkylamino-groups, and that similar behaviour is shown by a solvent-complexed monoalkylamino-derivative. In solvents of low polarity the unfavourable steric interaction of the nitro- and monoalkylamino-substituents in a planar conformation is offset by their conjugative interaction and the further stabilization afforded by hydrogen bonding between them; however, in more polar solvents both the nitro- and monoalkylamino-groups, freed of the constraint of the intramolecular hydrogen bond, rotate from the ring plane with consequent relief of steric strain.

In this connection, it was recently observed [5] that the rate constants of hydrolysis of 3-nitro-2-pyridylalanylglycine, despite the smaller basicity of the heterocyclic nitrogen atom, are significantly greater than those of 5-nitro-2-pyridylalanylglycine; this has been ascribed to steric hindrance in the ground state and, more tentatively, to a stabilization of the transition state by interaction of the amino-hydrogen atom with the oxygen atom of the vicinal nitro-group. With this in mind, it was also demonstrated that the use of 2-fluoro-3-nitropyridine presents significant advantages, when compared with Sanger's 1-fluoro-2,4-dinitrobenzene, in the determination of *N*-terminal amino-acids in peptides and proteins.[6]

In view of these facts we have investigated in detail the u.v.–visible absorption and c.d. properties of 3-Npy-amino-acids and compared them with those of analogous heteroaromatic derivatives; we believed that this approach might shed light on their structural and related stereochemical features.

EXPERIMENTAL

Materials.—All chemicals used were of analytical reagent grade. The samples of L-amino-acids, dinitrofluorobenzene and 2-chloro-3,5-dinitropyridine were purchased from Fluka (Switzerland); all these compounds were used without further purification. 2-Fluoro-3-nitropyridine and 2-fluoro-5-nitropyridine were synthesized as previously described.[7] The amino-acid derivatives were prepared in the usual manner.[6,8,9]

Methods.—M.p.s were determined with a Kofler hot-stage apparatus. The pH values of buffered solutions were measured at 25° with a Sargent–Welch PBX pH-meter previously calibrated at pH 4 and 7. U.v. and visible spectra were measured with a Hitachi–Perkin-Elmer 139 single-beam spectrophotometer fitted with a temperature-regulated cell holder. C.d. spectra were obtained from a Cary 60 automatic recording spectropolarimeter equipped with a Cary CD attachment. Cylindrical fused quartz cells with 0·05—1·0 cm path length were used. The data

† 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.

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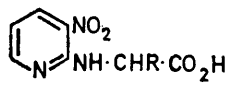
are expressed: (i) in terms of [θ], the molar ellipticity, defined as $[\theta] = 3300\Delta\varepsilon = 3300\ (\varepsilon_L - \varepsilon_R)$ deg cm^2 dmol^{-1}, where $(\varepsilon_L - \varepsilon_R)$ is the difference between the molar extinction coefficients for left- and right-handed circularly polarised light; and (ii) in terms of the dichroic optical density $\Delta A = A_L - A_R$, or of circular dichroic absorption $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, according to the equation $\Delta A = \Delta\varepsilon cd/M$, where c is the concentration (g l^{-1}), d is the cell path (cm) and M is the molecular weight. U.v. and c.d. spectra were recorded immediately after the preparation of solutions. Dioxan and cyclohexane were of spectroscopic grade (Merck).

RESULTS AND DISCUSSION

The absorption spectra of 3-Npy-amino-acids (1) in weakly alkaline media show a band in the 300—500 nm region centred near 420 nm, associated with the *ortho*-nitropyridylamino-chromophore. The corresponding band of the 5-Npy-amino-acids (2), associated with the



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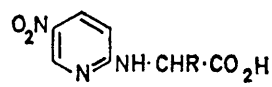

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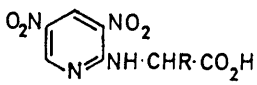

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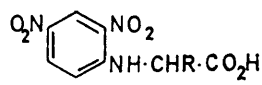

(4)

5-nitro-2-pyridylamino-chromophore is centred at 370 nm. The molar absorption coefficients at $\lambda_{max.}$ for 3-Npy-derivatives are notably lower than those of the isomeric 5-Npy-derivatives, particularly in the case of 3-Npy-proline.[6] This phenomenon is explained on the basis of a considerable steric inhibition of resonance and is accompanied by a large shift of wavelength maximum. Furthermore, the spectra of Dnpy-amino-acids (3) and Dnp-amino acids (4) show an intense band near 340 nm with a shoulder in the visible region.[10-13]

It is possible that the propensity for strong interaction of water molecules with the nitro-group might partially counterbalance the tendency towards non-planarity and impart particular rigidity to the structure of the 3-Npy-amino-acids through the existence of cyclic molecular complexes in which the water molecules bridge the monoalkylamino- and nitro-groups.[4] These added restrictions would drastically reduce the number of the possible conformational isomers. Visible absorption spectra of 3-Npy-amino-acids in water solution at various temperatures (20—60°) do not show any variation, suggesting a kind of effective rigidity within the chromophore. On this basis, a solvent-induced shift in the absorption of 3-Npy-amino-acids would be expected; this would also be anticipated from the high polarity of the ground state of these compounds.[14,15] In fact, 3-Npy-L-valine shows its long-wavelength absorption maximum at 390 nm in dioxan–cyclohexane solution. This property makes these chromophores promising environmentally sensitive ' reporter groups,' [16]

TABLE 1

C.d. data of *N*-(3-nitro-2-pyridyl)amino-acids [a]

L-Amino-acid	$\Delta A_{max.} \times 10^2$	L-Amino-acid	$\Delta A_{max.} \times 10^2$
Alanine	+1·54	Proline	−7·70
Leucine	+1·66	Arginine	−0·62
Isoleucine	+1·45	Lysine [c]	+0·73
Valine	+1·19	Ornithine [c]	+1·32
Serine	+1·04		−7·35
Threonine	+1·61	Diaminobutyric acid [c]	−0·81
Aspartic acid	+0·97		
Asparagine	+0·57	Phenylglycine [d]	−5·11
Glutamic acid	−0·20	Phenylalanine	−2·36
Glutamine	−1·01	Histidine	−1·48
Methionine	−0·89	Tyrosine	−3·41
S-Methylcysteine	−1·17	Tryptophan	−3·32
Cysteine [b]	−0·88		

[a] Overnight in 5% $NaHCO_3$–EtOH (4 : 1); amino-acid concentration 0·1 mol l^{-1}; excess of 2-fluoro-3-nitropyridine; 1 mm cell. [b] Dimethyl ester derivative. [c] Di-3-Npy-derivatives; the positive value reported for the ornithine derivative refers to the longer wavelength Cotton effect. [d] Measured for the D-enantiomer; sign reversed for convenience of comparison.

which can provide absorption spectral evidence relating to conformational changes of proteins in solution.

In view of the lower molar absorption coefficients of *ortho*-nitro-derivatives, we have examined in detail the chirospectroscopic properties of 3-Npy-amino-acids above 400 nm. The reported ΔA values (Table 1) have only relative significance, since the 3-Npy-amino-acids were studied directly in reaction mixtures; however, in every case, completion of reaction was tested spectrophotometrically. The results can be summarized as follows: (i) 3-Npy-L-amino-acids carrying chromophores absorbing at higher wavelength than 200 nm in their side chains have negative Cotton effects at 410—425 nm with the exception of 3-Npy-L-aspartic acid and 3-Npy-L-asparagine; (ii) 3-Npy-L-amino-acids which do not have chromophores absorbing at higher wavelength than 200 nm in their side chains present positive Cotton effects. The reported experimental data additionally suggest that the absolute value of the negative Cotton effect depends on the number of methylene groups which separate the chromophore in R from the asymmetric carbon atom (compare 3-Npy-L-methionine with 3-Npy-*S*-methyl-L-cysteine, and 3-Npy-L-phenylalanine with 3-Npy-L-phenylglycine). Furthermore, the orientation and distance of the chromophore of the amino-acid side-chain R, relative to the heteroaromatic chromophore, seem to be responsible for the inversion of sign observed

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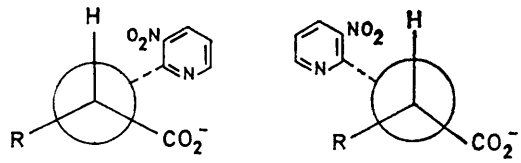
in going from L-aspartic acid (or L-asparagine) to L-glutamic acid (or L-glutamine), and for the complex pattern shown by the $N(\alpha)N(\omega)$-di-3-Npy-derivatives of L-lysine, L-ornithine, and L-diaminobutyric acid. Finally, in the case of 3-Npy-L-proline, the inversion of sign of the c.d. band (observed also for acetoacetyl-,[17] *N*-oxido-2-pyridyl-,[18] and thiobenzoyl-L-proline[19]) should be interpreted in the light of its probable adoption of a sterically more favoured conformation. In this conformation the relationship between the hetero-aromatic chromophore and groups at the asymmetric centre is inverted relative to that of 3-Npy-amino-acids carrying an alkyl group in their side-chain.

In view of the extremely low steric requirements of the hydrogen atom of the asymmetric carbon centre in comparison with those of amino-acid side-chain R and CO_2^- substituents, it is reasonable to assume that the conformational isomers of lower energy of 3-Npy-amino-acids would be (A) and (B). The ellipticity value and



sign of the circular dichroic bands appear to be governed by the proportions of conformational isomers (A) and (B) in the rotameric equilibrium mixtures, which are dependent (i) on the relative bulkiness of R and CO_2^-, and (ii) on the presence of groups in R which could interact with the nitro-heteroaromatic system repulsively or attractively.

The presence of aromatic chromophores in R must also be taken into account to explain the experimental data. In fact the strong Cotton effects observed for the 3-Npy-derivatives of the aromatic amino-acids (a consequence of π–π interaction) provide a striking contrast to the curves displayed by the other 3-Npy-amino-acids. This suggests that the difference is not solely the result of steric effects. These findings parallel those previously reported.[19-22]

A further control on the intensity and sign of the Cotton effect might be exercised by unsymmetrical twisting of the 3-nitro-2-pyridylamino-chromophore induced by a neighbouring asymmetric centre, *i.e.* the left- and right-twisted conformers might have unequal populations owing to interaction of the nitro-group with substituents on the asymmetric carbon atom. However, insofar as the population of conformers with nitro- and monoalkylamino-groups out of the plane of the pyridine ring is unknown, it is impossible to estimate quantitatively the contribution to magnitude and sign of the Cotton effects of the left- and right-twisted conformers.

The pH effect on c.d. $\lambda_{max.}$ and related ellipticity values was also investigated. Between pH 4 and 9·2, both parameters appear to remain substantially unchanged for all 3-Npy-amino-acids tested. Below pH 4 protonation of both carboxylate[11,17] and pyridyl[6,15] groups causes the ellipticity values at 418 nm to decrease; this is paralleled by the appearance of a new optically active band in the near u.v. region.

For comparison with the chirospectroscopic properties of 3-Npy-L-phenylalanine, we have studied the c.d. bands of 5-Npy-, Dnpy-, and Dnp-L-phenylalanine at about pH 9 in the 300—500 nm region. The results are shown in Table 2. It appears that the c.d. band I, centred at 405—425 nm, has a negative sign, whereas band II, centred at 330—370 nm, has a positive sign. In the case of Dnpy- and Dnp-L-phenylalanine, band I, which is covered in the u.v. absorption spectra because of the much more intense molar extinction coefficients of band II, stands out clearly in the c.d. spectra. We conclude that the most useful chromophoric derivative, as far as the problem of configurational assignment of amino-acids is concerned, is the 3-Npy-, since (i) 5-Npy-derivatives show much higher absorption and hence much more unfavourable $\Delta\varepsilon/\varepsilon$ ratios; and (ii) Dnpy- and Dnp-derivatives, in addition to possessing the drawback discussed for 5-Npy-derivatives, could present complications arising from the presence of bisignate curves. Furthermore, the chemical behaviour of 2-fluoro-3-nitropyridine,[6] along with the spectral and optical rotatory properties of the 3-Npy chromophore, suggest a rapid and simple method of correlating the sign of the 420 nm c.d. band and the absolute configuration of *N*-3-Npy-terminal amino-acids of peptides after cleavage of the first peptide bond under mild acidic conditions. The usefulness of the method is also apparent in view of the fact that the 3-nitro-2-pyridylamino-chromophore exhibits its long-wavelength optically active transition in a spectral range which is transparent for all the natural amino-acids.

TABLE 2

C.d. data of nitroaromatic *N*-substituted derivatives of L-phenylalanine [a]

Derivative	Band I	Band II
3-Npy	Negative (420 nm)	
5-Npy		Positive (350—370 nm) [b]
Dnpy	Negative (408 nm)	Positive (332 nm)
Dnp [c]	Negative (422 nm)	Positive (348 nm)

[a] 300—500 nm region, pH 9·1. [b] Broad band. [c] Two bands of opposite sign, centred at 440 and 360 nm, have been also observed in the c.d. spectrum of *N*-(β-2,4-dinitroanilinoethyl)-*NNN'N'N'*-pentamethylpropane-1,3-diammonium dibromide bound to DNA (E. J. Gabbay, *Biochemistry*, 1971, **10**, 1665).

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