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Chiroptical Probes for Configurational Analysis of α-Amino Acids[1]

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In the last fifteen years, ORD and CD techniques have been widely employed for purposes of investigating the conformations assumed by oligopeptides[2,3], poly-α-amino acids[3,4] and biologically active peptides[3,5,6], greatly contributing to the solution of the problem of their tridimensional architecture. In the field of α-amino acids, a major application of ORD and CD is represented by the investigation of configurations of their asymmetric carbon atoms in α-position. This latter approach appears to be of considerable interest, since natural α-amino acids, although found in the great majority of cases as L-configurational isomers, often occur as D-isomers, particularly in peptide antibiotics and in cell-wall material. This article reports the results of the application of CD to configurational analysis of α-amino acids, since this technique is to be preferred to ORD because it not only represents a more highly refined method but furnishes the maximum of useful information from the experimental data.

1. *Naturally occurring* D-*amino acids*

Although there is at present considerable doubt concerning the occurrence of D-amino acids in proteins, mainly due to the methods of hydrolysis and isolation which do not eliminate the danger of racemization[7], the presence of D-amino acids in a variety of microorganisms in both the free state and in peptides has been soundly demonstrated[8,9]. A number of antibiotics (e.g., polymyxins, gramicidins, tyrocidins, actinomycins, bacitracins) contain D-amino acids which form peptide bonds with L-amino acids. Antibiotics possessing D-amino acids may act as inhibitors of bacterial cell-wall synthesis by competing with normal D-amino acid substrates. Thus, D-cycloserine inhibits the enzymatic racemization of alanine and the synthesis of the dipeptide D-alanyl-D-alanine. In addition, the D-amino acids of antibiotics and cell-walls could impart a measure of protection to these molecules and structures by rendering them less susceptible to attack by peptidases. In this connection, it is of interest that the cellular glutamic acid of *B. subtilis* is of the L-configuration, while that of the capsule is predominantly D. There is now evidence for the occurrence of D-amino acids in insects. Thus, D-alanine has been found in the blood of butterfly larva *Danaus plexippus*. On the contrary, there is as yet no clear-cut proof for the presence of D-amino acids in mammalian organisms; the finding of D-pyroglutamic acid in human urine may be tentatively ascribed to the presence of D-glutamic acid in the cells of bacterial flora or in a dietary constituent. To conclude, the significance of the natural occurrence of D-amino acids is difficult to evaluate and awaits future explanation, but their presence in nature provides at least a reason for the existence of enzymes specific for the D-series. Many of the observations in this area point to yet undiscovered enzymatic reactions and may provide new ideas on regulation of cellular processes.

2. *CD configurational analysis of α-amino acids in the free state*

Among the several physico-chemical techniques employed to determine the configuration of α-amino acids in the free state, CD is particularly advantageous in that it provides the answer very rapidly, requiring smaller amounts of material. On the other hand, the enzymic methods are both sensitive and simple and they have been extensively applied. They are, however, limited to the determination of the configurations of those amino acids that are substrates for the enzyme employed. α-amino acids exhibit 2 optically

[1] The following abbreviations were used in the text: DNP, dinitrophenyl; DNPyr, 3,5-dinitro-2-pyridyl; 3-NPyr, 3-nitro-2-pyridyl; 5-NPyr, 5-nitro-2-pyridyl.

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active electronic transitions above 200 nm, which can readily be investigated by means of modern CD instrumentation. In fact, recent improvements in commercial apparatus permit the examination of CD spectra to 185 nm. These transitions are: a) the $n \rightarrow \sigma^*$ transition of the amino chromophore, which involves promotion of one electron from the lone-pair orbital of nitrogen atom to the lowest excited state, the anti-bonding orbital σ^*; and b) the $n \rightarrow \pi^*$ transition of the carboxyl chromophore, which involves promotion of one electron from the lone-pair orbitals of carbonyl oxygen to the lowest excited state of carboxyl group, the anti-bonding orbital π^*. The CD Cotton effect, associated with the $n \rightarrow \sigma^*$ transition of the amino group, is usually weak and disappears on protonation[10]. Therefore, on the basis of the physico-chemical properties of α-amino acids, one could examine this transition only in alkaline solutions, where the equilibria [equation (1)] are shifted to the right.

$$^{+}NH_3\text{—}CH(R)\text{—}COOH \rightleftarrows {}^{+}NH_3\text{—}CH(R)\text{—}COO^- \rightleftarrows NH_2\text{—}CH(R)\text{—}COO^- \quad (1)$$

On the other hand, the investigation of the CD band associated with the $n \rightarrow \pi^*$ transition of the carboxyl chromophore might definitely give more satisfactory results as far as the configurational analysis of α-amino acids in the free state is concerned. This intense CD band, whose pH-dependent location falls into the 200–215 nm region, is positive for L-amino acids and negative for D-amino acids[11]. Recently it has been demonstrated that carboxylic acids exhibit an additional CD band at about 240–250 nm, much weaker than that apparent at 210 nm and of opposite sign[10]. These findings have been interpreted assuming that both dichroic bands are associated with the $n \rightarrow \pi^*$ transition of the carboxyl chromophore. The ionization of the carboxyl group appears to cause the disappearance of the weak $n \rightarrow \pi^*$ band of lower energy. Thus, in the CD spectra of α-amino acids between 200–250 nm and in the pH region where their zwitterionic species prevail, a single Cotton effect is present at about 200 nm, whose sign provides the optical configuration of the compound under examination. The same relationship between sign of CD band at 210 nm and optical configuration holds also in the case of N-alkyl-α-amino acids[11]. However, a number of α-amino acids possess chromophores in their side-chains as well, which give rise to Cotton effects above 200 nm[3,6]. Among the more common α-amino acids, histidine shows a CD band at 213 nm, phenylalanine at 217 and at about 260 nm, unionized tyrosine at 225 and 275 nm, tryptophan at 218 and at about 280 nm and cystine at 220 and 260 nm. Consequently, in these cases, for purposes of configurational assignment one must refer to their complete dichroic pattern in the 200–300 nm region.

3. *CD configurational analysis of α-amino acids in peptides*

In this section we report the chirospectroscopic properties of the reaction products of α-amino acids with halo-nitroaromatic compounds and isothiocyanates. These reagents are those mainly in use to determine the primary structure of naturally occurring peptides. The reaction products have been investigated to ascertain if CD technique, along with the chemical procedures reported, can be employed to determine simultaneously both the nature and the optical configuration of α-amino acids in peptides. These chromophoric derivatives of the amino function allow one to be acquainted readily and unequivocally with the configuration of the asymmetric carbon atom since they: a) possess a chromophore which is optically inactive by nature and becomes optically active as a result of the presence of the asymmetric carbon atom in its vicinity, b) absorb in the near-UV and VIS region (300–450 nm), i.e. where the side-chain chromophores of α-amino acids are transparent, and c) exhibit rather high values for the Kuhn dissymetry factor g ($g = \Delta\varepsilon/\varepsilon$), which permit one to obtain CD spectra with favourable signal to noise ratios.

3A. *CD configurational analysis of N-terminal α-amino acids*

Recently a novel procedure for the determination of the N-terminal amino acids in peptides and proteins has been described[12] (Scheme 1). The free amino moieties are allowed to react with 2-fluoro-3-nitropyridine in slightly alkaline solution; the resulting derivatives are easily hydrolyzed in dilute hydrochloric acid to give the corresponding 3-NPyr-amino acids, which can be separated and estimated spectrophotometrically. The advantages of this reagent, when compared with Sanger's fluoro-dinitrobenzene[13], are many-fold. In particular, the presence of the aza-function has a relevant effect because of the anchimeric assistance of the heterocyclic nitrogen atom, and highly preferential cleavage of the peptide bond at the end of the chain is possible under mild conditions. In view of these facts, the CD properties of 3-NPyr-amino acids have been investigated[14], since this approach appeared to represent a promising method of configurational analysis of N-terminal α-amino acids. For the sake of comparison the UV-absorption and the

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[12] A. SIGNOR, L. BIONDI, A. M. TAMBURRO and E. BORDIGNON, Europ. J. Biochem. *7*, 328 (1969).
[13] F. SANGER, Biochem. J. *39*, 507 (1945).
[14] C. TONIOLO, L. BIONDI, D. NISATO and A. SIGNOR, J. chem. Soc., Perkin I, *1972*, 1179 and 1183.

optical rotatory properties of DNP-amino acids and of other nitropyridyl-amino acids, namely 5-NPyr-[12,14] and DNPyr-amino acids[14–17], have been reported.

The absorption spectra of 3-NPyr-amino acids in weakly alkaline media show a single absorption band in the 300–500 nm region centred near 420 nm[14]. Their molar absorption coefficients at λ_{max} are notably lower than those of isomeric 5-NPyr-amino acids (Figure 1). This phenomenon is explained on the basis of a considerable steric inhibition of resonance and is accompanied by a large shift of wavelength maximum. Since the 370 nm band, associated with the *para*-nitropyridyl-amino chromophore, is much higher than the 420 nm band, associated with the *ortho*-nitropyridyl-amino chromophore, it is conceivable that the spectra of DNP- and DNPyr-amino acids reveal a band in the near-UV with a shoulder in the VIS (Figure 1). In view of the lower molar extinction coefficients of *ortho*-nitro derivatives, the chirospectroscopic properties of 3-NPyr-amino acids have been examined in detail[14]; some representative examples are shown in Figure 2. The results obtained can be summarized as follows: a) 3-NPyr-L-amino acids, carrying chromophores absorbing higher than 200 nm in their side-chains, have negative Cotton effects at 410–425 nm; and b) 3-NPyr-L-amino acids, which do not have chromophores absorbing higher than 200 nm in their side-chains, present positive Cotton effects. The most suitable pH range for CD measurements was also determined. Between pH 4 and 9.2, the CD ellipticity values remain unchanged for all 3-NPyr-amino acids tested; below pH 4, protonation of both carboxylate and pyridyl groups causes the $\Delta\varepsilon_{max}$ values to decrease substantially (Figure 3). Parallel investigations performed in a number of organic solvents have shown that in all cases the $\Delta\varepsilon/\varepsilon$ values are largely lower than those observed in water at pH 8–9.

The CD properties of 3-NPyr-, 5-NPyr-, DNPyr- and DNP-L-phenylalanine, at about pH 9 and in the 300–500 nm region, have been compared. The results obtained show that the CD band centred at 405–425 nm has a negative sign, whereas the CD band centred at 330–370 nm has a positive sign. On these bases it is possible to conclude that the most useful of the chromophoric derivatives investigated seems to be 3-NPyr- since: a) the 5-NPyr-derivative shows much higher absorption and hence much more unfavourable $\Delta\varepsilon/\varepsilon$ ratios; and b) the DNPyr- and DNP-derivatives, in addition to the drawback above discussed for the 5-NPyr-derivative, could possibly present complications arising from the presence of bisignate curves.

The chemical behaviour of 2-fluoro-3-nitropyridine and 3-NPyr-amino acids, along with the spectral and optical rotatory properties of *ortho*-nitropyridyl-amino chromophore, allowed us to suggest a new method of correlating the sign of the 420 nm CD band and the

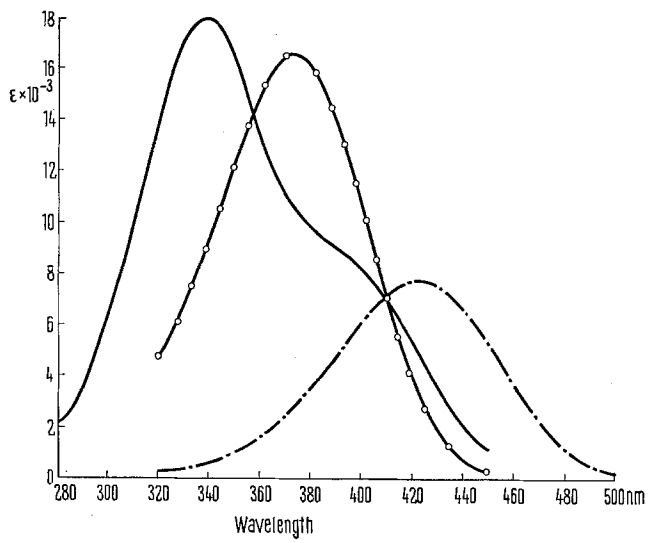

Fig. 1. UV-absorption spectra of 5-NPyr-L-valine dicyclohexylammonium salt (-○-○-); 3-NPyr-L-valine (–.–.–) and DNPyr-L-valine (—) in 1% $NaHCO_3$.

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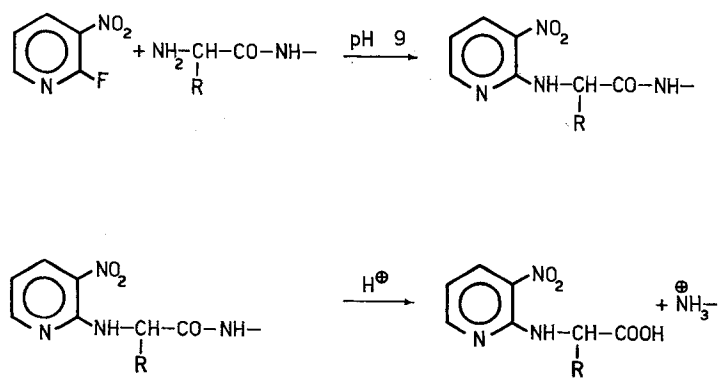
Scheme 1



optical configuration of N-terminal α-amino acids. Since the signs of CD bands of 3-NPyr-amino acids are dependent both on the absolute configuration of the α-carbon atom and on the side-chain, it is evident that for configurational assignments one must be aware of the nature of the side-chain. This method can be employed unequivocally also in consideration of the fact that the possible reaction with other nucleophilic groups present in peptides (particularly mercapto- and ε-amino-groups) does not induce any variation in the sign of CD bands at 420 nm. In fact, 3-NPyr-amino acids, prior to CD measurements, are separated by chromatographic techniques from the modified peptides. In the experimental conditions proposed, the dissymmetry factors $\Delta\varepsilon/\varepsilon$ for 3-NPyr-amino acids are high enough to allow the determination of CD curves by using a $10^{-4}M$ solution of the chromophoric derivatives. In addition, preliminary CD investigations of dansyl-amino acids[18] did not give encouraging results; these compounds show only one broad CD band above 300 nm, centred at 345–365 nm at pH 8.4, whose intensity (and hence the $\Delta\varepsilon/\varepsilon$ ratio) is very low[19]. Consequently, the use of the 3-NPyr-chromophore appears to be more suitable than the dansyl-chromophore for assigning the optical configuration of the N-terminal amino acids of peptides.

3B. *CD configurational analysis of sequences of α-amino acids*

The Edman degradation[20] is at present the method exclusively employed in sequential analysis of peptides (Scheme 2). Recently the original method, which uses phenylisothiocyanate as reagent, has been improved by using methylisothiocyanate[21]. The latter reagent is more soluble in water, making it possible to perform the reaction in aqueous solution without resorting to the addition of organic solvents. In this section we report the chirospectroscopic properties of methylthiohydantoins derived from free α-amino acids and peptides; they have been studied[22] in order to ascertain whether the method could be applied in connection with the modified Edman degradation technique, to determine sequentially the configuration of α-amino acid residues in peptides. The UV absorption spectra of methylthiohydantoins present two maxima near 265 and 235 nm with a shoulder at about 320 nm. Methylthiohydantoins show a Cotton effect at 302–315 nm at acidic pH, which is positive for the L-amino acids and negative for D-amino acids (Figure 4). The location of the band in a region of low absorption, and its red shift on changing the solvent from water to chloroform, suggest that it is associated with an $n\rightarrow\pi^*$ transition within the acylthioureido chromophore. Analogous results have recently been found for phenylthiohydantoins derived from α-amino acids[23]. It is important to note that the sign of the CD Cotton effect at 310 nm of methylthiohydantoins is not dependent on the nature of the side-chain, but is a function exclusively of the optical configuration of the asymmetric α-carbon atom. A further interesting observation concerns the sign of CD bands of methylthiohydantoins, which do not invert on changing the solvent from water to chloroform, as has already been shown for other thiocarbonyl compounds[24]. Therefore, since $\Delta\varepsilon_{max}$ in chloroform are of the same order of magnitude as those in acidic aqueous solution, and since it is necessary to remove the drawback that the

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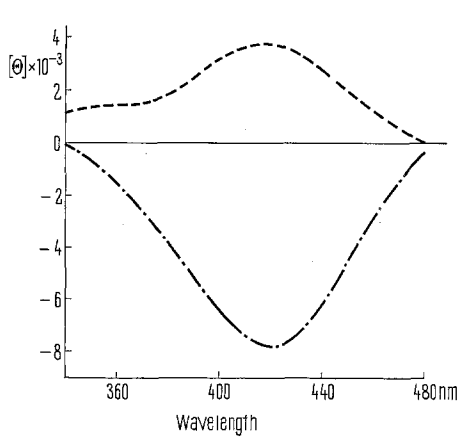


Fig. 2. Circular dichroism spectra of 3-NPyr-L-valine (– – –) and 3-NPyr-L-phenylalanine (–.–.–) at pH 9.1.

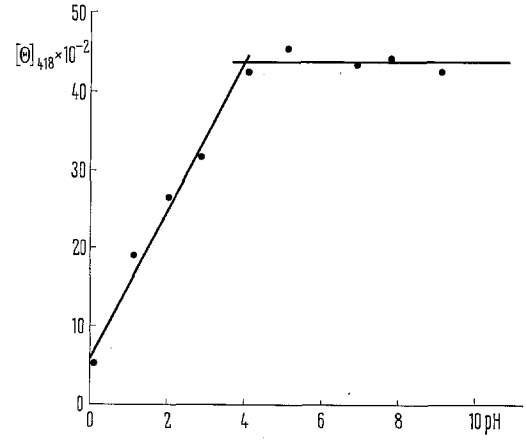


Fig. 3. Plot of molar ellipticity at 418 nm of 3-NPyr-L-valine versus pH.

reaction of methylisothiocyanate with –SH groups eventually present in peptides could produce, it is advisable to extract the methylthiohydantoins with chloroform and to make the CD measurements in this solvent. This method of configurational analysis has been tested on a couple of diastereomeric dipeptides containing alternate L- and D-alanine residues. In both cases methylthiohydantoins exhibited CD Cotton effects of alternate sign and corresponding to those expected on the basis of the optical configuration of the asymmetric α-carbon atom. Therefore the sign of the CD band rapidly supplies configurational assignment of sequences of α-amino acids in peptides.

The sensitivity of the method is satisfactory, since it allows the use of about $2\times10^{-4}M$ solution of the chromophoric derivative. The main limitation is due to the discrete tendency of methylthiohydantoins to racemize. In the Edman degradation, racemization can be explained on the basis of the formation of isomeric 4-thiazolinones as intermediates (Scheme 2); this phenomenon, if not carefully controlled by avoiding high temperatures and pH values during the coupling reaction, could lower the sensitivity of the proposed method.

4. *Configurational analysis of α-amino acids in peptides. Chirospectroscopic properties of side-chain chromophoric derivatives*

The CD technique also offers additional possibilities for the configurational analysis of α-amino acids in peptides; in fact it is possible to take advantage of the chirospectroscopic properties of chromophoric derivatives of functional groups present in side-chains.

The chemical reagents which can be used in this approach, besides giving rise to chromophoric derivatives possessing the characteristics previously reported, must also react whith peptides in selective and non-racemizing conditions. In addition, the sign of the CD band due to the resulting chromophore must be compared to that of a model compound in the same solvent, provided that the Cotton effect is not influenced by the secondary structure of the peptide. As an example of this approach, the chirospectroscopic properties of chromophoric derivatives of –SH groups and their possible application to configurational analysis of cysteine in peptides have been examined. The major

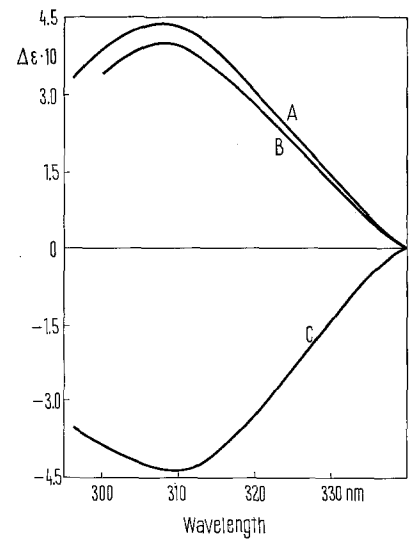
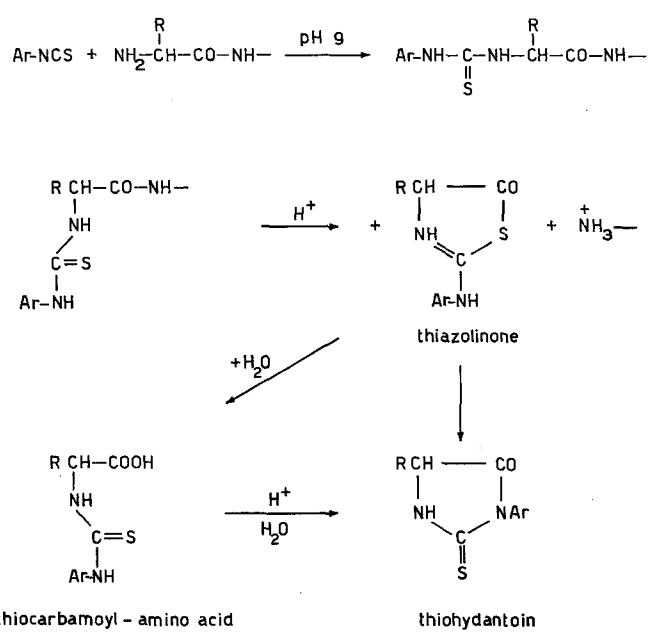


Fig. 4. Circular dichroism spectra of methylthiohydantoins derived from L-leucine (A), L-phenylalanine (B) and D-leucine (C).

Scheme 2



role of the configuration of cysteinyl residues in natural products has been illustrated by a recent aspect of stereospecificity-bioluminescence relationships[25]. Furthermore it was demonstrated that a limited number of antibiotics, namely malformin[26], siomycin[27] and thiostrepton[28] contain D-cysteine residues, and penicillin one residue of penicillamine (β,β-dimethyl-D-cysteine)[9].

The reaction products of cysteinyl compounds with 2-fluoro-3-nitropyridine and methylisothiocyanate were recently investigated for configurational analysis of the –SH function[14,22]. The cysteinyl peptides react quantitatively with 2-fluoro-3-nitropyridine in a few minutes, whereas the reaction with the amino groups is not yet detectable (Figure 5). These CD findings confirm that selective modification of –SH relative to the other nucleophilic groups in peptides is readily achieved in favourable conditions[29]. The UV-VIS-absorption spectra of S-3-NPyr-L-cysteine derivatives at pH 9 exhibit a band near 365 nm, associated with the *ortho*-nitropyridyl-thio chromophore. Moreover, in the CD spectra, a positive Cotton effect is apparent centred at about the same wavelength. As in the case of nitropyridyl-amino compounds (section 3A), S-3-NPyr-derivatives show much more favourable $\Delta\varepsilon/\varepsilon$ ratios in the near-UV region when compared with 5-NPyr-, DNPyr- and DNP-analogs, and hence are the most useful for configurational analysis. Since S-3-NPyr-derivatives, when measured at different pH and

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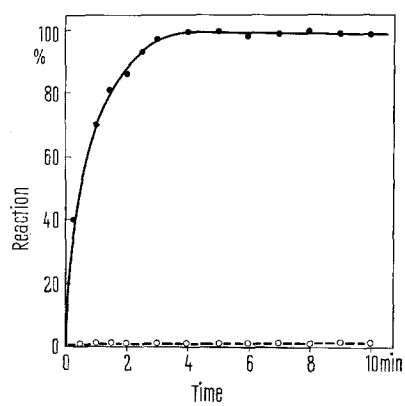

Fig. 5. Reaction rate of glutathione (1.4 mg/ml) and 2-fluoro-3-nitropyridine (6 mg/ml) in 5% $NaHCO_3$-ethanol (4:1), followed at 365 nm (–×–×–) and at 425 nm (–○–○–); 1 mm cell, sensitivity 0.1, temperature 22 ± 0.5 °C.

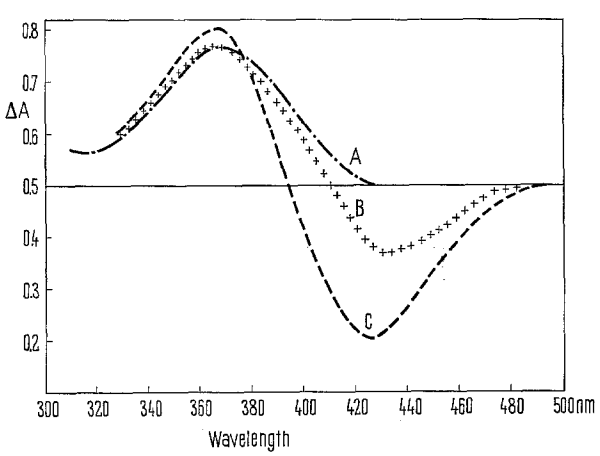

Fig. 6. Circular dichroism spectra of reaction mixtures of 2-fluoro-3-nitropyridine (6 mg/ml) with glutathione (1.4 mg/ml) in 5% $NaHCO_3$-ethanol (4:1), after 20 min (A) and 330 min (B), and with L-cysteine (0.67 mg/ml) after 20 min (C); 1 mm cell, temperature 22 ± 0.5 °C. Curve B in the region 410–500 nm has been amplified 2.5 times.

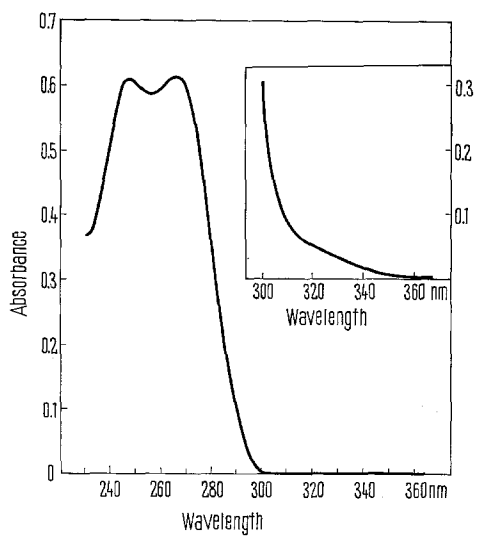

Fig. 7. UV-absorption spectrum of S-methylthiocarbamoyl-glutathione at pH 4.0.

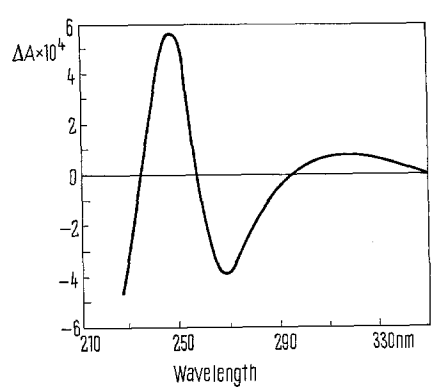

Fig. 8. Circular dichroism spectrum of S-methylthiocarbamoyl-glutathione at pH 4.0; $10^{-2}M$, 0.5 mm cell.

in different solvents, do not show $\Delta\varepsilon/\varepsilon$ values consistently higher than those shown at pH 9, these reaction conditions are the most suitable for CD measurements. When free L-cysteine was made to react with 2-fluoro-3-nitropyridine, the positive band at 367 nm and the negative band at 425 nm increase simultaneously as a consequence of S→N aryl migration (Figure 6). As a result of these studies it may be postulated that, when thiol and amino moieties are in a suitable relative position, they react as a common functional group with electrophilic compounds. This implies that a selective modification of –SH groups is not possible in these cases. However, this does not complicate the configurational assignments, since both CD bands appear to be diagnostic of the optical configuration of cysteine residues.

On the other hand, if methylisothiocyanate is allowed to react at pH 4–6 with cysteinyl peptides, selective S-methylthiocarbamoylation takes place[22]. The derivatives exhibit a weak UV-absorption band at about 325 nm in water, along with bands of greater complexity at about 270 and 250 nm (Figure 7). When the dithiocarbamate group is in a dissymmetrical environment, these transitions present optical activity; Figure 8 illustrates the CD curve of S-methylthiocarbamoyl-glutathione at pH 4. The sign of the 320 nm CD band is strictly solvent-dependent, as shown in other thiocarbonyl compounds[24]; therefore it is not automatically transferable to organic or aqueous-organic solutions. Hence, since the 320 nm CD band presents a high dissymmetry factor, its sign at pH 4–6 can be used to determine the optical configuration of the α-carbon atom of cysteine in peptides, being positive for L-cysteinyl derivatives and negative for D-derivatives. In the experimental conditions employed, no S→N shift of –C(=S)-NHMe group has been observed.

Therefore, on the basis of chirospectroscopic properties of S-3-NPyr- and S-methylthiocarbamoyl-cysteinyl derivatives, it is possible to conclude that both chromophoric derivatives are extremely valuable, so far as the configurational assignment of cysteine in naturally occurring peptides is concerned.

Zusammenfassung. Es wird eine neue Methode der stufenweisen Konstitutionsaufklärung von Polypeptiden vorgeschlagen, mit einer gleichzeitigen Bestimmung der Aminosäuren-Konfiguration. Es ergab sich, dass man im Falle der freien Aminosäuren die Chromophorengruppe des Carboxyls, gegebenenfalls auch jene der Seitenketten benützen kann. Für die Peptide sind neue Chomophorenderivate N-endständiger Aminogruppen und der SH-Funktion des Cysteins vorgeschlagen worden.

SPECIALIA

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On the Biosynthesis of Neoflavanoids

In continuing our studies on the biosynthesis of 4-phenyl coumarins[1] (neoflavanoids[2]) we investigated the biogenetic relationship between calophyllic acid (Ia)[3] and inophyllolide[3] (II), two minor constituents of *Calophyllum inophyllum* (Guttiferae) seeds. We wished to establish whether it is the acid (Ia) or the lactone (II) which is biosynthetized first. The comparison of specific activities after incorporation of a common radioactive precursor should resolve this question, since the intermediate should be the more radioactive compound[4].

We therefore administered 3-^{14}C phenylalanine, an established precursor of the C_9 unit of 4-phenyl coumarins[4], to young shoots of *C. inophyllum* in 2 separate experiments (lasting 1 and 2 weeks, respectively). Calophyllic acid (Ia) and inophyllolide (II) were isolated by repeated chromatography and crystallized to constant radioactivity[5]. The results (dpm/mM) of both experiments are given in the Table. They reveal an approximately 8-fold specific activity for (Ia) [counted as its more soluble methyl ester (Ib)] than for (II). This suggests that calophyllic acid (Ia) is the biogenetic precursor of inophyllolide (II)[6].

In order to control the above results we fed, in a short-term experiment (24 h), U-^{14}C isoleucine, a very efficient precursor of the 2,3-dimethyl chromanone ring[7]. Again,

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