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Note

2,4-Dinitrophenylpyridium chloride, a novel and versatile reagent for the detection of amino acids, primary and secondary amines, thiols, thiolactones and carboxylic acids during planar chromatography

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Hemlock alkaloids[1] and nicotinamide[2] have been detected with 1-chloro-2,4-dinitrobenzene and primary aromatic amines[3] and nitrofurans[4] by a photochemical reaction with pyridine but 2,4-dinitrophenylpyridium chloride (DPPC) has not been previously used as a chromatographic detecting reagent.

DPPC can be considered a charge-transfer complex, in which the electron-withdrawing dinitrophenyl moiety causes a decrease of electron density within the pyridine ring, rendering its α-carbon atoms susceptible to nucleophilic attack[5–7], as shown in Fig. 1. In this paper, we report the first simple synthesis of DPPC and its subsequent use in differentiating between the various classes of nucleophiles, colorimetrically and fluorimetrically.

EXPERIMENTAL

Unless stated otherwise, all procedures were carried out at 20–23°C.

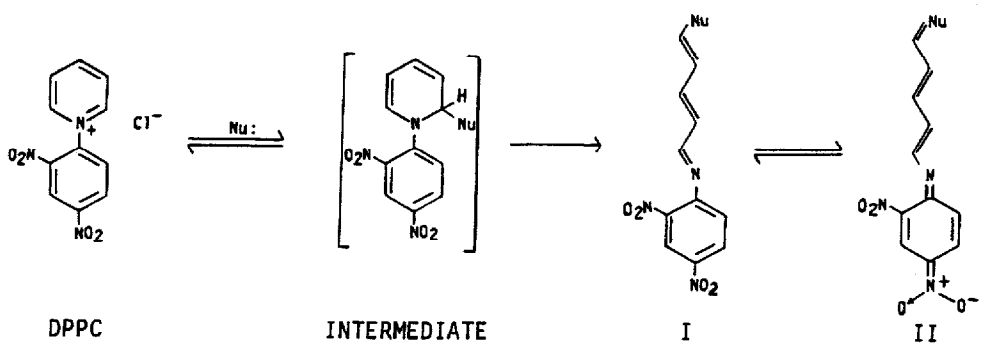

Fig. 1. The general reaction of DPPC with nucleophiles (Nu:) to give the mixed glutaconic aldehyde derivative (I) or the aci-nitro form (II) of I.

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Materials

Chemicals were mainly supplied by BDH (Poole, U.K.). Ammonia solution (sp.gr. 0.88) and most of the solvents used for chromatography were of analytical-reagent grade like the starting materials for DPPC synthesis but butanone, butan-1-ol, 2-methyl-propan-2-ol and propan-2-ol were ordinary-reagent grade. Glass-distilled water was used throughout.

Preparation of DPPC

1-Chloro-2,4-dinitrobenzene (0.2 g) in 2.5 ml acetone was mixed with 0.4 ml pyridine and left to stand for 72 h in a stoppered Pyrex tube in the dark. The solid formed was filtered by suction, washed with 5 ml ice-cooled acetone and air dried. Crystal colour: light beige brown. Yield, 0.135 g (48%): M.p., 187–193°C (Lit. 183–200°C)[18]. Solid DPPC slowly dissociates on standing so the reagent was kept in methanolic solution (200 mg l^{-1}), which is stable for at least 0.5 year at 20°C in darkness.

TLC

All samples chromatographed were made up at 25 m*M* and stored at −20°C. Amino acids and carboxylic acids were dissolved in 10% (v/v) aqueous propan-2-ol; asparagine, tyrosine and cystine were solubilised by the further addition of HCl to a final concentration of 0.05 *M*. Amines were dissolved in 70% (v/v) aqueous methanol containing 0.025 *M* HCl if as free bases.

Aliquots (0.2–0.6 μl) were spotted 1 cm from the edge of Merck cellulose (Art. 5578, DC Plastikrolle, 100 μm thickness) or Merck silica gel (Art. 5553, DC Alufolien, 200 μm thickness, without fluorescent indicator) layers.

Ascending chromatography was in one of the following solvents: (I) 2-methyl-propan-2-ol–butanone–propanone–methanol–water–ammonia (40:20:20:1:14:5, v/v) or (II) butan-1-ol–propanone–acetic acid–water (35:35:10:20, v/v) in a glass chromatographic tank. These were selected as examples of acidic and basic solvents already established for the separation of amino acids[8,9].

After the solvent front had migrated 9 cm, plates were dried in an air current, 15 min for solvent I and 45 min for solvent II. When all the R_F values were initially low, layers were chromatographed a second time in the same direction and re-dried.

Detection procedure

Plates were sprayed with DPPC in methanol (200 mg l^{-1} for cellulose and 100 mg l^{-1} for silica gel) until they just appeared translucent. After drying in an air stream for 10 min, the reaction was initiated by placing them in a sealed glass chromatographic tank (30 × 27.5 × 8.7 cm), which had been thoroughly rinsed with a mixture of 8 ml methanol and 2 ml triethylamine, the excess having been poured away. Methanol was essential for full colour differentiation and development of a yellow background.

When the plates were yellow–yellow brown, after *ca.* 1 min for cellulose and 8 min for silica gel, they were removed and examined. This was designated stage A. The layers were then returned to the alkaline atmosphere for a further 15 min, and either examined immediately or after air drying for 30 min (stage B). They were then kept in a dark cupboard for 24 h before recording the fluorescences that had normally developed (stage C).

TABLE I

AMINO ACIDS

R_F values were determined at a loading of 2.5 nmol. Key: BH = bleached; Bg = beige; Br = brown; Dk = dark; G = green, Lt = light; ND = no absorbance or fluorescence at a loading of 25 nmol; Or = orange; Pk = pink; L = lilac; Pu = purple; Rd = red; W = white; * = colour of *fluorescence* as recorded under 366 nm UV light. (nmol) = Detection limits in nmol at the given stage. Where, as in Tables II and III, colours were identical at stages A and B, sensitivities, except those marked [o], were recorded at stage A. When sensitivity was based on fluorescence, irradiation was at 366 nm. Sometimes (Table II) the fluorescence intensity increased when the layers were kept a further 14 days in darkness and this is recorded as an extension of the stage C results.

Compound[a]	*Cellulose, solvent I run* ×2				*Cellulose, solvent II*			
	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*
1 4-Aminobutyric acid	17	Br–Rd (1.0)	Lt Rd (0.75)	Bg–Or* (1.0)	56	Or–Br (1.0)	Lt Br (0.5)	Bg–Or* (0.75)
L-Arginine–HCl	5	Or–Br (7.5)	Lt Or (2.5)	Bg* (0.5)	15	Lt Or-Br (2.5)	BH (2.5)	Bg* (0.75)
L-Aspartic acid	2	Or–Br (15.0)	BH (1.0)	Bg–Or* (0.75)	21	Lt Or (10.0)	BH (2.5)	Bg–Or* (0.75)
L-Asparagine	9	Or–Br (10.0)	BH (1.0)	Bg–Or* (0.75)	15	Or–Br (15.0)	BH (5.0)	Bg–Or* (0.75)
L-Cysteic acid	4	Br Rd (5.0)	Or Br (5.0)	Bg–Or* (1.0)	8	Or–Br (5.0)	Lt Rd (1.0)	Bg–Or* (0.75)
L-Cystine	1	Lt Or (5.0)	BH (2.5)	Bg–Or*(2.5)	4	Or–Br (1.0)	Or (2.5)	Bg–Or* (2.5)
Glycine	11	Rd (0.75)	Rd (0.5)	Bg–Or* (0.25)	26	Rd (0.5)	Rd (0.75)	Bg–Or* (0.75)
L-Histidine	18	Br Rd (5.0)	BH (1.0)	Bg–Or* (0.75)	16	Or–Br (2.5)	BH (1.0)	Bg Or* (0.75)
L-Leucine	54	Rd (0.5)	Lt Br (2.5)	Bg–Or* (0.75)	80	Lt Rd (0.75)	Lt Rd (1.0)	Bg–Or* (0.75)
Isoleucine	54	Rd (0.5)	Lt Br (2.5)	Bg–Or* (0.75)	79	Lt Br (0.75)	Lt Rd (1.0)	Bg–Or* (0.75)
L-Lysine–HCl	11	Lt Rd (1.0)	Rd (0.5)	Bg–Or* (0.75)	14	Or–Br (2.5)	BH (1.0)	Bg–Or* (0.25)
L-Methionine	37	Or–Br (5.0)	Br (1.0)	Bg–Or* (2.5)	57	Or–Br (1.0)	Lt Rd (2.5)	Bg–Or* (5.0)
L-Methionine sulphone	23	Or–Br (5.0)	Lt Or (1.0)	Bg–Or* (0.75)	31	Or–Br (2.5)	Or (2.5)	Bg–Or* (0.75)
5-Methyl-L-Tryptophan	64	Br (1.0)	Y (1.0)	Y* (0.75)	79	Or–Br (1.0)	Y (1.0)	Y* (0.75)
L-Phenylalanine	58	Or–Br (1.0)	Lt Rd (5.0)	Bg–Or* (0.75)	70	Or–Br (1.0)	Lt Rd (7.5)	Bg–Or* (0.75)
L-Serine	22	Rd (1.0)	Lt Rd (5.0)	Bg–Or* (1.0)	22	Or–Br (2.5)	Lt Rd (2.5)	Bg–Or* (0.75)
L-Tyrosine	29	Br (2.5)	BH (0.75)	Bg–Or* (0.75)	58	Or–Br (1.0)	BH (1.0)	Bg–Or* (0.75)
L-Tryptophan	48	Lt Br (2.5)	BH (1.0)	Bg–Or* (0.75)	58	Or–Br (2.5)	Lt Br (7.5)	Bg–Or* (0.75)
2 L-Azetidine-2-carboxylate	17	Pu (0.15)	Dk Br (0.1)	Y–G* (0.25)	36	Pu (0.2)	Dk Br (0.1)	G* (0.25)
4-Hydroxy-L-proline	15	Pu (0.2)	Pu (0.15)	G* (0.25)	23	Pu (0.25)	Dk Br (0.15)	G* (0.5)
4-Hydroxymethyl-L-proline	17	Pu (0.1)	Dk Br (0.05)	G* (0.25)	32	Pu (0.25)	Dk Br (0.1)	Y–G* (0.25)
4-Methyl-L-proline	37	Pu (0.05)	Dk Br (0.025)	G* (0.25)	63	Pu (0.15)	Dk Br (0.05)	G* (0.25)
N-Methyltaurine	39	Rd–Pu (0.5)	Dk Rd (0.25)	Y–G* (0.5)	37	Rd–Pu (0.25)	Dk Rd (0.2)	Y–G* (0.5)
D-Pipecolate–HCl	42	Pu (0.75)	Dk Br (1.0)	Lt G* (1.0)	52	Pu (1.0)	Dk Br (0.75)	Lt G* (0.75)
L-Proline	23	Pu (0.05)	Dk Br (0.025)	G* (0.25)	41	Pu (0.15)	Dk Br (0.05)	G* (0.25)
Sarcosine	21	Pu (0.15)	Br–Pu (0.1)	Y–G* (0.25)	39	Pu (0.25)	Dk Br (0.2)	Y–G* (0.25)

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

TABLE II

AMINES

For details, see Table I.

Compound[a]	*Cellulose, solvent II*			*Silica gel, solvent II*			
	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*	*(nmol)*
1 Putrescine–di-HCl	10	Rd (0.5)[0]	Bg–W* (2.5)	6	Or–Br (1.0)	W* (1.0)	
Benzylamine	74	Or–Br (0.5)	Or* (5.0)	62	Or–Br (0.15)	W* (2.5)	
Ethanolamine	34	Or–Br (5.0)	Bg–Or* (2.5)	25	Or–Br (2.5)	W* (5.0)	
3-Methoxy-4-hydroxyphenylethylamine–HCl	63	Or–Br (2.5)	Bg–Or* (5.0)	61	Br (0.2)	W–Pu* (1.0)	→ Pu* (0.1)
m-Tyramine	68	Br (1.0)	W* (1.0)	66	Or–Br (0.2)	W–L* (0.15)	→ L* (0.025)
o-Tyramine–HCl	73	Br (1.0)	Bg–Or* (2.5)	69	Br (0.25)	W* (0.5)	→ Or* (0.2)
p-Tyramine–HCl	69	Or–Br (0.5)	Bg–Or* (2.5)	66	Or–Br (0.5)	W* (0.25)	→ Or* (0.15)
2 Cytisine	38	Dk Rd–Pu (0.15)[0]	G* (0.5)	15	Rd–Pu (0.025)	G* (0.5)	→ G* (0.25)
Diethanolamine	39	Pu (0.1)[0]	G* (0.5)	29	Dk Rd (0.5)	G* (2.5)	
N-Methylbenzylamine HCL	79	Pu (0.1)	G* (0.75)	59	Rd–Pu (0.075)	G* (1.0)	→ G* (0.75)
N-Methyl-3-methoxy-4-hydroxyphenylethylamine-HCl	66	Pu (0.05)	Y–G* (1.0)	56	Rd–Pu (0.1)	W* (0.5)	→ Or* (0.1)
Morpholine	45	Rd–Pu (0.5)	Y–G* (1.0)	31	Dk Rd (1.0)	Y–G* (2.5)	
Piperazine–di-HCl	6	Pu (0.05)	G* (0.5)	2	Pu (0.2)	G* (1.0)	
Spermine–tri-HCl	2	Rd–Pu (0.2)	Y–G* (0.75)	1	Dk Rd (0.25)	W–G* (0.75)	

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

RESULTS AND DISCUSSION

Tables I–IV show the sequential colours/fluorescences that developed after reaction with DPPC, together with minimum detectable quantities for four classes of compounds.

Table I indicates that all primary amino acids tested initially gave brown spots which had developed bleached centres by stage B if loadings exceeded 5 nmol: they were totally bleached by stage C but could still be detected, normally at enhanced sensitivity, by their beige–orange fluorescence under UV light. Primary amines (Table II) reacted similarly but were better recorded at stage A/B. In contrast, secondary amino acids and secondary amines first gave a purple colour which formed a yellow centre at loadings of > 2.5 nmol (stage B): finally a yellow spot was associated with a green fluorescence. Table II confirms that initial colour and the wavelength of fluorescence do provide a reliable way of distinguishing between amino and imino groups.

The best discrimination between the two types of fluorescence is obtained under 254 nm UV light, while the greatest sensitivity is given by irradiation at 366 nm. Fluorescence intensity often increases with time, at least on silica gel, doubling on average when stage C plates are kept a further 14 days in darkness: the effect is recorded in Table II where it leads to a substantial increase in sensitivity for individual primary and secondary amines. Attempts to stimulate fluorophore production by heat and/or by increased exposure time to triethylamine–methanol were unsuccessful. Replacing triethylamine with ammonia solution actually diminished subsequent fluorescence.

A cellulose chromatogram at stage A, if kept in air, will absorb moisture; its background will darken; purple spots will become dark brown and other colours will similarly degenerate leading to a loss of sensitivity. However, layers at stage A can be stabilised by sandwiching them immediately between pairs of glass plates, pre-washed in acetone.

The pH of the chromatography solvent sometimes has a minor effect on colour

TABLE III

AROMATIC AMINES

For details, see Table I.

Compound[a]	*Cellulose, solvent II*			*Silica gel, solvent II*		
	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A/B (nmol)*	*Stage C (nmol)*
p-Phenylenediamine–di-HCl	56	Pu (2.5)[0]	Or* (1.0)	54	Br-Pu (1.0)	Or* (0.75)
Sulphathiazole	85	ND	Lt Br (25)	84	ND	Lt Rd–Br (20)
2-Aminoimidazole sulphate	48	ND	Lt Br (20)	55	ND	Br (25)
p-Aminobenzoate	91	Br (10.0)	Or* (2.5)	92	Br (2.5)[0]	Or* (1.0)
p-Anisidine	77	Br (10.0)	Pk–Or* (2.5)	71	Br (2.5)[0]	Or* (1.0)
2 Diphenylamine	98	ND	ND	92	ND	Y (15.0)
Skatole	99	ND	ND	96	ND	Br (25.0)

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

quality (Table I) but none at all on the appearance of the fluorescence at stage C. Table I also implies that sensitivity is unimpaired by residues from either solvent.

No data are given for amines run in solvent I on cellulose as the corresponding chromatograms streak extensively. However, Tables II and III do show results for amines run on both cellulose and silica gel in solvent II. Silica gel may produce minor changes in absorbance/fluorescence characteristics and on average doubles sensitivity towards primaries and halves that for secondary amines.

Tables II and III only contain a selection of the amines tested. *n*-Butylamine, cadaverine, diethylamine, di-*n*-butylamine, octopamine, 2-phenylethylamine, piperidine, pyrrolidine, spermidine and synephrine also react with DPPC giving the colours/fluorescences predicted from their chemical structures: all were easily visible at 5 nmol.

Unlike most equivalent reagents, DPPC will detect amino and imino groups linked directly to aromatic rings (Table III) but sensitivities are often very poor: indeed aniline, N-methylaniline and indole have been excluded from the table because they did not react visibly at 25 nmol. Even those aromatic amines that were detectible often gave no fluorescence at stage C, just a yellow or brown colouration.

Amides and amidines seem not to react: urea and guanidine were certainly negative at 25 nmol.

The DPPC reagent is bleached at all stages by thiol compounds like mercaptoethanol and thioglycolic acid. It will detect the non-volatile thiols, dithiothreitol and cysteine on Whatman 3MM paper at levels down to 0.5 and 2.5 nmol cm^{-2} respectively. However, it has been impossible to assay sensitivities under the standard conditions adopted here because of the chemical changes that occur during chromatography. For example, all preparations of cysteine tested (3 of the free base and 4 of the hydrochloride) showed two spots as recorded in Table IV. The slower is almost certainly cysteine, by comparison with its characteristics in Table I, but relative R_F values suggest that the faster and major component is cystine thiolactone. Certainly

TABLE IV

THIOLS AND CARBOXYLIC ACIDS

For details, see Table I

Compound[a]	*Cellulose, solvent I run* $\times 2$				*Cellulose, solvent II run* $\times 2$			
	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*	hR_F	*Stage A (nmol)*	*Stage B (nmol)*	*Stage C (nmol)*
L-Cysteine-HCl	33	BH (0.75)	BH (0.75)	BH (2.5)	63	BH (1.0)	BH (1.0)	BH (2.5)
	1	ND	ND	Bg-Or*	4	ND	ND	Bg-Or*
DL-N-Acetylhomocysteine thiolactone	98	BH (20)	BH (10)	BH (10)	95	BH (15)	BH (5.0)	BH (5.0)
DL-Malic acid	3	BH (0.75)	BH (10)	BH (15)	82	BH (7.5)	BH (10)	BH (15)
Glycolic acid	18	BH (1.0)	BH (15)	BH (20)	84	BH (7.5)	BH (15)	BH (20)
L-Pyroglutamic acid	23	BH (2.5)	BH (15)	BH (20)	87	BH (10)	BH (15)	BH (20)
Itaconic acid	6	BH (0.75)	BH (5.0)	BH (10)	98	BH (5.0)	BH (10)	BH (15)
Succinic acid	4	BH (0.75)	BH (5.0)	BH (10)	96	BH (5.0)	BH (10)	BH (15)

[a] Numbers 1 and 2 refer to primary and secondary nitrogen, respectively.

DPPC will detect the much more stable acetylhomocysteine thiolactone, albeit poorly.

Carboxylic acids also bleach the reagent (Table IV) but this time the visible effect declines rapidly as the pH increases from stage A to B.

DPPC has considerable value as a functional group reagent. Initial colour and fluorescence wavelength at stage C certainly provide a reliable way of distinguishing between primary and secondary nitrogen, while the bluish fluorescence developed by 3-methoxy/hydroxy primary phenylethylamines 14 days after stage C may be a specific test for this class of compound. It is even possible to distinguish between thiols and carboxylic acids by monitoring their bleaching reactions carefully.

However, DPPC often lacks sensitivity. It would not, for example, be the reagent of choice for detecting thiols or carboxylic acids. Even for primary amino compounds it is generally less sensitive than ninhydrin[10], *o*-phthalaldehyde[11] and fluorescamine[12,13] by factors of *ca.* 4, 8, and 2–8 respectively. This probably overstates its disadvantage as we have measured sensitivities under conditions that give good chromatographic separation and it is competitive for individual compounds like *m*-tyramine and 3-methoxy-4-hydroxyphenylethylamine. Nevertheless it is most satisfactory for the detection of aliphatic and alicyclic secondary amino compounds which it can generally do at the 0.2-nmol level. Here competing reagents include vanillin[14] and nitrobenzoyl chloride[15], but these will only detect NH in amino acids, not in amines. Nitroprusside–acetaldehyde[16] detects the same classes of secondary nitrogen as DPPC but its sensitivity towards the secondary amines listed in Tables II and III was at least 10 times less when tested after solvent 2 on silica gel. The fluorescamine–taurine[17] spray has a broader specificity than any reagent so far mentioned as it will also reveal NH linked to an aromatic ring but its sensitivity is sometimes poor, >5 nmol for proline and is impaired by both acidic and alkaline solvent residues.

As for the chemistry of these reactions, R_2NH groups allow greater delocalisation of electrons over the conjugated system than do RNH_2 groups, yielding the red–purple aci-nitro structure (Fig. 1) at a considerably lower pH. The carboxyl groups of amino acids also promote delocalisation to a lesser degree: hence the tendency of amino acids to give reddish colours at stage A. The structure of the fluorophores at stage C is unclear at present.

Thiols react with the dinitrophenyl moiety, dissociating the DPPC and so preventing colour formation at all stages. Pyridine is known to catalyse this type of acylation[6].

Finally, bleaching by carboxylic acids is due to their buffering properties: they resist the increase in pH when the plates are exposed to triethylamine, so temporarily prevent colour development.

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