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Note

Formation of diastereoisomeric derivatives from the enantiomers of the antitumour agent cyclophosphamide by reaction with 1-phenethyl alcohol, and their separation by thin-layer chromatography

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During an investigation into the metabolism of the enantiomers[1] of the antitumour agent cyclophosphamide {2-bis(2-chloroethyl)amino-2*H*-1,3,2-oxazaphosphorine-2-oxide, I} a method was required for monitoring the enantiomeric composition of cyclophosphamide recovered from the urine of patients given either the racemate or an enantiomer. Separable diastereoisomeric derivatives of cyclophosphamide have been prepared previously from the racemate by derivatisation at N-3 of the oxazaphosphorine ring using (—)-(*S*)-α-naphthylphenylmethylsilyl chloride[2]. The present procedure depends on the stereospecific cleavage of one of the P–N linkages by a chiral alcohol, 1-phenethyl alcohol, to form diastereoisomeric derivatives in which the chiral centres are separated likewise by only one heteroatom.

Treatment of cyclophosphamide with sodium hydride under anhydrous conditions affords a bicyclic product (II) containing a strained P–N bridge bond[3]. In the presence of a suitable nucleophile, release of strain by cleavage of one of the P–N linkages would be anticipated. Accordingly, when cyclophosphamide (racemate or enantiomers) was allowed to react with the sodium salts of (+)- or (—)-1-phenethyl alcohol evidence for the reaction sequence I–IVa (Fig. 1) was obtained.

EXPERIMENTAL

Racemic cyclophosphamide was purchased from Koch-Light Labs. (Colnbrook, Great Britain) and (+)- and (—)-1-phenethyl alcohol ($[\alpha]^{20}$ +39.57° and —42.95°, respectively) from Norse Labs., Los Angeles, Calif., U.S.A. (+)-(*R*)- and (—)-(*S*)-cyclophosphamide were prepared by a published procedure[1].

Chromatography

Thin-layer chromatography was carried out on glass plates (5 × 20 cm) coated with a 0.25-mm layer of Kieselgel G. Separated components were visualized by exposure to iodine vapour or by spraying with 1% ethanolic 4-(4-nitrobenzyl)-

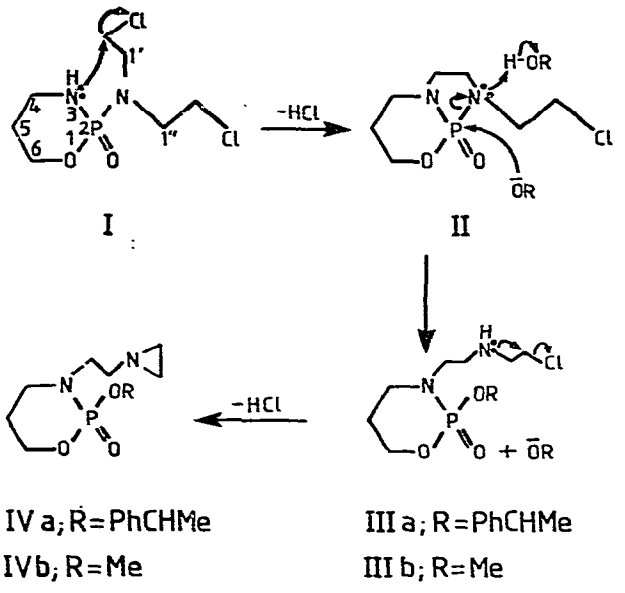

Fig. 1. Scheme for the reaction of cyclophosphamide with (a) 1-phenethyl alcohol and (b) methanol.

pyridine[4]; heating at 150° for 30 min then spraying with 1% ethanolic potassium hydroxide solution (blue spots on white ground).

Reaction of cyclophosphamide in (a) phenethyl alcohol and (b) methanol

(a) To a solution (0.1 ml) prepared by dissolving sodium (46 mg) during 2 h in a mixture of benzene and (—)-1-phenethylalcohol (0.5 ml each) was added cyclophosphamide (racemate or either enantiomer; 1 mg). The progress of the reaction was monitored by thin-layer chromatography in chloroform–ethanol (9:1).

(b) A solution of cyclophosphamide (racemate, 10 mg) in methanolic sodium methoxide (0.5 *M*, 1 ml) was heated under reflux. The reaction was followed by thin-layer chromatography in chloroform–methanol (9:1).

Mass spectrometry

The products were eluted from thin-layer chromatograms using methanol. The concentrated eluates were applied to the direct insertion probe. Mass spectra were determined using an AEI-MS 12 spectrometer at an ionizing voltage of 70 or 12 eV, a trap current of 100 μA and an ion-source temperature of 90–110°.

RESULTS AND DISCUSSION

The reaction of cyclophosphamide in phenethyl alcohol was complete within 5 min. The racemate afforded two products, R_F 0.28 and 0.33 which gave very similar mass spectra consistent with diastereoisomeric structures IVa. Evidence that the principal ion *m/e* 150 (Fig. 2) was formed by loss both of the phenethyl and aziridinomethyl moieties was obtained by carrying out the same reactions with two (racemic) tetradeuterated analogues of cyclophosphamide. The products given by a

ring (4,6-d_4) deuterated analogue[5] gave a base peak at *m/e* 154 (molecular ion $M^{+\cdot}$ at *m/e* 314) whereas the base peak from the side-chain (1′, 1″)-labelled congener[6] was again at *m/e* 150, showing that this ion retains the oxazaphosphorine ring, but not the methylene groups originally adjacent to the exocyclic N-atom in the cyclophosphamide molecule.

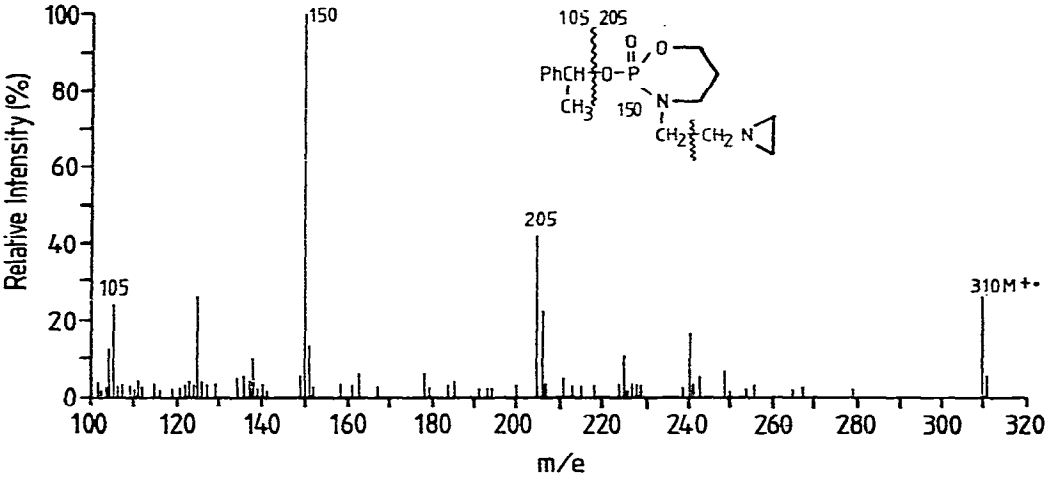

Fig. 2. Mass spectrum of the product of R_F 0.33 in chloroform–ethanol (9:1) formed from (−)-phenethyl alcohol and racemic cyclophosphamide. This spectrum was recorded at an ionizing voltage of 12 eV to maximize the relative intensity of the molecular ion: at 70 eV this fell to 7.3%.

Each enantiomer of cyclophosphamide, when treated as above with (−)-phenethyl alcohol afforded one product only; the (+)-(*R*)-form gave the diastereoisomer of IVa having the higher R_F value. The situation was reversed when the reaction was conducted using (+)-phenethyl alcohol. Hence the step (II → IIIa) in which a bond to the chiral phosphorus atom is cleaved, occurs without detectable racemization. The question of whether complete inversion, or retention of configuration is responsible for the optical purity of the products from the cyclophosphamide enantiomers remain open, although inversion seems more likely, on the basis of mechanisms which have been proposed for the alkaline hydrolysis of phosphoramidates[7].

The putative intermediates (II and IIIa) were not detected, even on chromatograms developed after reaction for only 30 sec, at which time cyclophosphamide was still the major component. However an analogue (IIIb) of one such intermediate was isolated during the slower reaction between cyclophosphamide and methanolic sodium methoxide, which failed to react during 24 h at room temperature, but which afforded after 15 min heating under reflux two principal products of R_F 0.18 and 0.26, having mass spectra (70 eV) corresponding to structures IIIb and IVb respectively. Thus, the former gave a molecular ion at *m/e* 256 (6.7% relative intensity) with the accompanying ^{37}Cl-containing satellite at *m/e* 258, and a base peak at *m/e* 164 that can be ascribed to the ion $[M - CH_2NHCH_2CH_2Cl]^+$. The latter product also gave the base peak ion at *m/e* 164, and the molecular ion at *m/e* 220 (6.9%,

relative intensity) appropriate to IVb. The intermediate IIIb was completely converted into IVb after 2 h reaction.

Neither the products (IVa) from 1-phenethyl alcohol nor the product IVb from methanol was isolable in bulk. Attempts at column chromatography led to decomposition, presumably owing to the lability of the aziridino moiety.

The reaction of cyclophosphamide with the optical isomers of 1-phenethyl alcohol affords a simple method of monitoring enantiomeric homogeneity. Thus when the above-described reaction was carried out using either enantiomer of cyclophosphamide containing 5% (w/w) of its antipode (total 1 mg) the minor component was easily detected by exposure to iodine vapour or by the Epstein test following thin-layer chromatography of one-tenth of the reaction mixture. The same fraction of a reaction mixture from 100 μg of racemic cyclophosphamide also afforded readily detectable bands, showing that the method could give at least a qualitative indication of enantiomeric composition (and hence of any stereoselectivity in metabolism) using a quantity of cyclophosphamide which would be easily recoverable[6] from a few millilitres of urine from patients given a conventional therapeutic dose of the racemate.

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