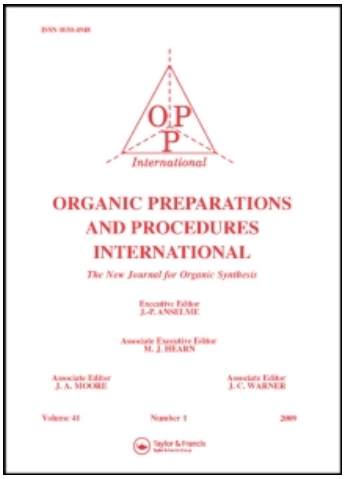

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COMPARISON OF SYNTHETIC ROUTES TO DL-CANALINE

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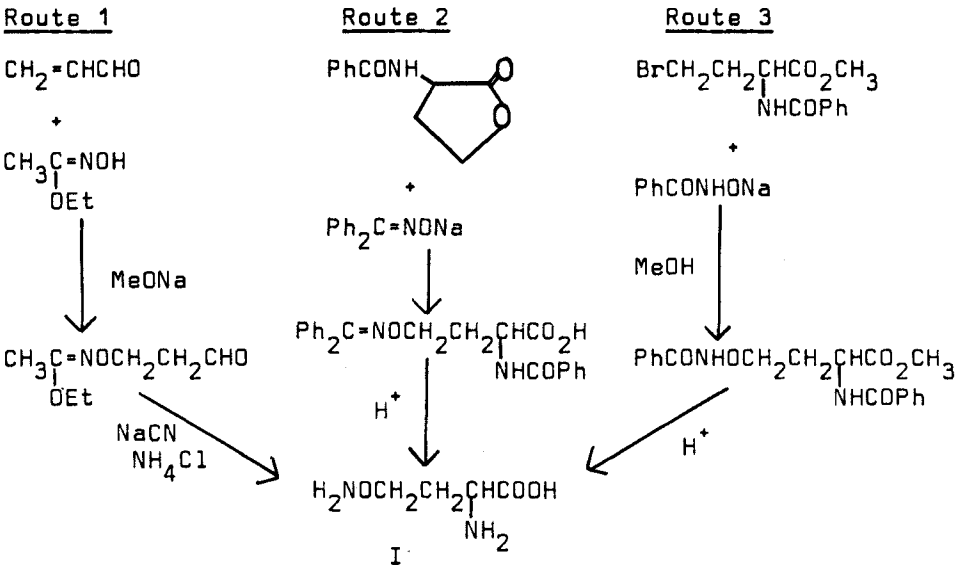
Canaline (α-amino-γ-aminooxy butyric acid), a natural product derived from canavanine by the enzyme arginase,[1,2] is a strong inhibitor of many B_6-vitamin dependent enzymes.[3,4] A lower homolog of canaline, α-amino-β-aminooxy propionic acid, has been succesfully used in biospecific chromatography of alanine aminotransferase.[5] Since substrates of other B_6-enzymes resemble canaline they could be purified with a gel to which canaline is attached. Although a number of syntheses have been published,[6-9] synthetic canaline is not commercially available. Therefore three different routes of the synthesis were tested as shown in Scheme 1.

Route 1. Acrolein method[8]

Coupling of oximino acetic acid ethyl ester to acrolein was unsuccesful in several solvents (methanol, DMSO, DMF and pyridine) and at temperatures ranging from -5 to 100° using $NaOCH_3$ or Et_3N as base; acrolein diethyl acetal was equally unsuccesful. Possible yields below about 10% were undetectable for in this case the reaction mixture was analyzed directly by NMR spectrometry. The acrolein method would have certain advantages in a large-scale preparation of DL-canaline. A related way from acrolein to DL-canavanine[10] should be

easily modified to the synthesis of DL-canaline.



Scheme 1

Route 2. Lactone fission method[8]

Difficulties also arose in the lactone fission. Dissolving an amount of sodium equivalent to benzophenone oxime in N-methyl pyrrolidone took an unreasonably long time if the oxime was well dried over P_2O_5 *in vacuo*. While 75% of the sodium dissolved readily, the remainder could only be dissolved by adding more benzophenone oxime. The reaction temperature of the lactone fission was critical; vigorous refluxing was required so that the reaction mixture turned black. If the reaction mixture was brownish, only little canaline was detected. The maximum yield of canaline obtained was 25% as measured from the reaction mixture. Since the sticky substance obtained from the acidification of the water was hard to purify, it was hydrolyzed and the hydrolyzate filtered and evaporated. The crude solid was purified by crystalli-

zation from ethanol and acetone as reported,[9] but was shown to be canaline acetoxime by titration and NMR spectra. Hydrolysis to canaline was achieved by heating with 18% HCl.

Route 3. Alkyl bromide method[7]

The aminooxy group is commonly introduced into alkyl chains by a substitution reaction of hydroxamic acid salt on alkyl halides.[11-13] The canaline syntheses of this type[6,7] are probably best carried out via α-(benzamido)-γ-bromo butyric acid ester.[7] The coupling reaction gave yields comparable to those of Knobler and Frankel,[7] but only after some modifications. The reaction in methanol produced canaline in 50% yield, but in ethanol only 30% of the theoretical (measured from the reaction mixture). A higher reaction temperature (63^{o}-67^{o}) and longer time were also necessary. Benzhydroxamic acid gave much higher yields than oximino acetic acid ethyl ester, hydroxyurethane or benzophenone oxime, all of which gave yields below 10%. When oximino acetic acid ethyl ester was used as the solvent the yield was 32% (hydroxyurethane gave 7% in the same way). Crystallization of the intermediate product failed; only a milky suspension in water was achieved.

Canaline synthesis via the alkyl bromide was the most reliable of those tested in the critical coupling step. Lactone fission and alkyl bromide methods gave pure canaline only with hydrolyzing the reaction mixture followed by ion-exchange chromatography[14] or precipitating canaline as its oxime.

EXPERIMENTAL

The starting materials were prepared according to literature procedures: benzophenone oxime,[15] oximino acetic acid ethyl ester,[16] hydroxyurethane,[11] benzhydroxamic acid,[17] α-amino-γ-butyrolactone·HBr,[18] methyl and ethyl α-benzamido-γ-bromobutyrate.[19] The oximes and hydroxamic acids were freshly prepared and stored at -20°. The purity of the starting materials was checked by NMR and IR spectra using 60 MHz Perkin-Elmer R 10 and Perkin-Elmer Model 337 spectrometers. Melting and boiling points were as reported in the literature.

Alkyl bromide method.[7] The biginning of the synthesis was made according to Knobler and Frankel.[7] A sealed flask which was magnetically stirred in a waterbath was used. The reaction time was 18 h at 67°. The mixture was then cooled, filtered and evaporated. The oil was hydrolyzed by 270 ml 18% HCl 3 h at 110°. The hydrolyzate was cooled and filtered to remove benzoic acid. After evaporation the oil was dissolved in abs. EtOH (50 ml). Et_3N was added dropwise until basic (pH=8). The mixture was kept overnight at -20° and the precipitate was collected. The crude canaline was dissolved in hot acetone. After cooling canaline precipitated as acetoxime. The solid was hydrolyzed to canaline with 50 ml 18% HCl 30 min at 100°. After evaporation canaline was achieved as very hygroscopic dihydrocloride. Yield 2.1 g (26%). Acetoxime: m.p. 211°. IR (cm^{-1}) 2100, 1625 ($-NH_3^+$), 1585 (COO^-), 1660 (C=N), 1515, 1420, 1355, 1330, 1075. NMR (ppm, δ-units) triplets 3.85, 4.2; quartet 2.2; singlet 1.9 (measured in D_2O, pD 8.0). Canaline: Dipolar ion m.p. 190-193° (dec.). IR (cm^{-1}) 2140, 1615, 1510, 1420, 1345, 1050. NMR quartet 2.15; two coalescing triplets 3.96, 3.85, 3.75, 3.69 (pD 8.0).

Lactone fission method.[8] The reaction was made according to Gilon et al.[8] The mixture was refluxed at 220° for 4 h, then concentrated in vacuo and poured into water (500 ml). Unreacted oxime was removed and the solution was again concentrated in vacuo. The oil was dissolved in 18% HCl (200 ml) and was kept at 110° for 3 h. The mixture was filtered and evaporated in vacuo. The purification was made as mentioned in the alkyl bromide method. Yield 2.4 g (11.5%). The NMR and

IR spectra were consistent for canaline.

Detection of canaline. Canaline was detected on Whatman no. 1 paper chromatography using descending elution with butanone: propionic acid: water (75:25:30). This solvent system was found to be better than those reported earlier.[2] The papers were sprayed with 0.1% ninhydrin plus 4% pyridine in acetone and kept at 60° for 30 minutes. The following R_f values and colors were obtained: canaline 0.72 (violet), canaline acetoxime 0.65 (violet), homoserine lactone 0.45 (yellow), homoserine 0.27 (violet). Spraying with 0.05 M pyridoxal-5'-phosphate in 0.5 M phosphate buffer pH 7 gave immediately a white spot (R_f=0.19) on yellow background for hydroxylamine. The amino acids gave dark yellow spots with this spray. The canaline spot also became slowly white.

Paper electroforesis. Paper electroforesis using 0.1 M sodium citrate buffer, pH 3.3 gave the following relative mobilities (ninhydrin spray): arginine 1, homoserine lactone 1.08, canaline 0.81, canaline acetoxime 0.45 and homoserine 0.42. The time was 4 h (8.5 V/cm).

Quantitation of canaline. The amount of canaline was measured in the following way: the reaction mixtures were evaporated *in vacuo* and then hydrolyzed with 18% HCl at 110° in sealed ampoules for 3 hrs. The amount of canaline in the hydrolysate reached maximum after 2-3 hrs. and stayed constant for at least 2 hrs. This behaviour indicates that the impurities in the reaction mixture do not significantly catalyze decomposition of canaline during the hydrolysis. Appropriate samples (containing 30-200 µg of canaline) were taken from the hydrolysate to be chromatographed as described above. The violet spots were cut out and dissolved in 2 ml of methanol and absorption at 570 nm was measured. Standards were made in the same way from commercial L-canaline (Sigma Chem. Co.).

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