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FACTORS INFLUENCING BY-PRODUCT FORMATION IN THE REACTION OF 1-FLUORO-2,4-DINITROBENZENE WITH AMINO SUGARS AND AMINODEOXYALDITOLS

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ABSTRACT

By-product formation, in decreasing order of severity, occurred during the reaction of 2-amino-3-*O*-[1-(*S*)-carboxyethyl]-2-deoxy-D-glucose (muramic acid), 2-amino-2-deoxy-D-glucose, and the corresponding aminodeoxyalditols with 1-fluoro-2,4-dinitrobenzene. The fine balance between incomplete derivatisation at pH 8.5 and the formation of by-products at pH 9.5 was best achieved by reaction in carbonate buffer (pH 9.0) in aqueous ethanol. Higher pH values or temperatures, and especially the use of tertiary amine buffers and dipolar aprotic solvents, increased by-product production. The implications for quantitative analysis of amino sugars are discussed.

INTRODUCTION

The *N*-(2,4-dinitrophenyl) (Dnp) derivatives of amino sugars have been used in qualitative[1] and quantitative analysis[2] and as synthetic intermediates[3]. Early separations involved crystallisation or relatively crude, chromatographic systems. The corresponding Dnp-aminodeoxyhexitols have been favoured for analysis by some workers[4], since they are less prone to degradation during column chromatography. In later experiments employing the superior resolving power of thin-layer chromatography (t.l.c.), crude reaction mixtures containing 2-deoxy-2-(2,4-dinitroanilino)-D-glucose (Dnp-D-GlcN) were shown to contain unknown by-products[2]. The present work arose from the preparation of Dnp-derivatives of 2-amino-3-*O*-[1-(*S*)-carboxyethyl]-2-deoxy-D-glucose (muramic acid; Mur) and the corresponding aminodeoxyalditol (muramitol; Mur_2). In both cases, t.l.c. on silica gel demonstrated that relatively large amounts of by-products were produced. Furthermore, both 2-amino-2-deoxy-D-glucose (D-GlcN) and 2-amino-2-deoxy-D-glucitol (D-$GlcNH_2$) also yielded by-products on reaction with 1-fluoro-2,4-dinitrobenzene (FDNB), though in smaller amounts. We now report on some of the factors that influence by-product formation and describe how this can be minimised.

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EXPERIMENTAL

Muramic acid was synthesised by the procedure of Osawa and Jeanloz[5], except that benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside was prepared by the method of Arendt *et al.*[6]; the superiority of the latter method over the procedure of Kuhn *et al.*[7] was confirmed. In a few experiments, commercial muramic acid (Sigma) was used for the reaction with FDNB, and these gave similar results.

Amino sugars were reduced using up to 4 mol of $NaBH_4$/mol of amino sugar for 0.5–1 h at 4° followed by 1.5 h at room temperature[4]. The process was monitored by t.l.c. in solvent *A*.

T.l.c. was performed on plates of silica gel containing a fluorescent indicator (Silica Gel 60 F254, Merck) by ascending development in the dark with *A*, toluene–methanol–glacial acetic acid (1:3:1); *B*, chloroform–methanol–glacial acetic acid (85:15:5); or *C*, chloroform–methanol–glacial acetic acid (95:5:1). Amino sugars and their alcohols were detected with ninhydrin or alkaline $AgNO_3$, and Dnp-compounds by illumination at 254 nm. Electrophoresis was performed at pH 6.5 in ammonia–0.15M aqueous acetic acid, using silica gel plates as support media. $DnpGlcNH_2$, DnpGlcN, and 2,4-dinitroaniline were used as uncharged markers to correct for endosmotic flow.

RESULTS AND DISCUSSION

The first step in examining the factors that lead to by-product formation was a comparison of conditions previously used for dinitrophenylation of amino sugars. These differed mainly in the buffers and solvents used. Since borate buffer[1] has been reported to catalyse degradation of the product[8], it was omitted from the present work, in order to diminish the number of variables.

Ethanol[9], acetone[3], or 1,4-dioxane–*N*,*N*-dimethylformamide[2] were used by previous workers to increase the rather low solubility of FDNB in the aqueous reaction medium. In the present work, mixtures containing either GlcN or Mur, or the corresponding aminodeoxyalditols, were prepared by using the same ratios of amino compound, buffer, water, organic solvent, and FDNB employed in these earlier reports. As well as using Na_2CO_3 with acetone[3], $NaHCO_3$ was also tested. In place of the 1,4-dioxane–*N*,*N*-dimethylformamide mixture, an equal volume of *N*,*N*-dimethylformamide (DMF) was used, since 1,4-dioxane tends to form peroxides that may interfere with recovery of Dnp-compounds.

Samples were removed after shaking at room temperature in the dark for 0.5, 1, 2, 4, 12, or 18 h. Separation by t.l.c. and visual inspection of chromatograms was very satisfactory for detecting both unreacted amino compounds, using solvent *A* (R_F values: GlcN = 0.46; $GlcNH_2$ = 0.19, Mur = 0.57, $MurH_2$ = 0.31), and Dnp-derivatives, using solvent *B* (see Table I). Reaction was usually completed in 2 h under mild conditions (aqueous, ethanolic sodium carbonate buffer, pH 9.0; at room temperature; with a 6–12 fold excess of FDNB). Each sample gave a spot

TABLE I

T.L.C. SEPARATION[a] OF BY-PRODUCTS FORMED DURING REACTION OF AMINO SUGARS AND THEIR ALCOHOLS WITH FDNB

Starting material	$R_F \times 100$
Glucosamine	0.27, 0.36, 0.44
Glucosaminitol	0.11, 0.16, 0.23, 0.39, 0.51
Muramic acid	0.26, 0.36, 0.44
Isomuramic acid	0.32, 0.45, 0.50
Muramitol	0.04, 0.11, 0.16, 0.23, 0.39, 0.51

[a]Separations were performed in Solvent *B*. The major products formed under mild conditions (*i.e.*, Dnp-GlcN, Dnp-$GlcNH_2$, Dnp-Mur, Dnp-Isomur, and Dnp-$MurH_2$) are underlined; the remainder are by-products.

corresponding to the Dnp-derivative desired, together with very fast-moving material ($R_F > 0.80$; mainly dinitrophenol and any unreacted FDNB). Increase of pH or temperature, or the presence of acetone or DMF, produced one or more by-product spots which occupied intermediate positions (see Table I) and were not present in controls that lacked the amino compound. In this way, the factors favouring complete conversion of the starting material with minimum formation of by-products were assessed. When conditions favoured formation of by-products, both Dnp-GlcN and Dnp-Mur gave products having R_F values of 0.36 and 0.44 in solvent *B* (see Table I); there is no evidence that either of the pairs of spots having the same mobilities are chemically identical. Rather surprisingly, since Dnp-aminodeoxyalditols have been considered to be more stable than the Dnp derivatives of the parent amino sugars[4], the factors described below which caused by-product formation in Dnp-GlcN and Dnp-Mur syntheses had similar effects in syntheses of Dnp-$GlcNH_2$ and Dnp-$MurH_2$, though to a lesser extent. Both aminodeoxyalditols gave by-products having very similar patterns of R_F values (see Table I), but, again, chemical identities cannot be assumed.

The tendency to form by-products decreases in the order Mur > $MurH_2$ > GlcN > $GlcNH_2$. The formation of Dnp-Mur always occurred concurrently with side reactions. With care, Dnp-$MurH_2$ could be made without detectable by-products and was the most sensitive indicator for unfavourable reaction conditions. There was little tendency for by-product formation when $NaHCO_3$ and ethanol were used. However, complete conversion of the starting material was difficult to achieve without a considerable excess of FDNB, in which case reaction with ethanol produced a substantial amount of 1-ethoxy-2,4-dinitrobenzene. This side-reaction could be avoided by using acetone as solvent. T.l.c. showed that by-product formation from the amino sugars was only a little worse with acetone, but the solution turned permanently brownish-yellow on mixing. As this effect probably reflects the well-known tendency of ketones to form coloured complexes of uncertain structure with aromatic nitro-compounds[10], acetone is best avoided.

With DMF as solvent, complete conversion of the starting material was rapidly attained, but by-products were always prominent. If ethanol or acetone was used under stronger conditions (*e.g.*, Na_2CO_3 rather than $NaHCO_3$, with overnight incubation at room temperature, or 38° for shorter periods), the pattern of spots resembled that produced in the early stages of the reaction when DMF was present. Apart, perhaps, from the fastest-moving by-products produced by long incubation in DMF, the solvent effects on by-product production appeared to the quantitative.

When the volatile buffer triethylamine carbonate (TEA-CO_2, pH 8.9) was used to avoid interference by salt in subsequent chromatography, the formation of faster-moving by-products was favoured. If both DMF and TEA were present during derivatisation of aminodeoxyalditols, side reactions were particularly severe, with an increase in the product having R_F 0.39 and the appearance of a series of extra products having R_F values between 0.51 and 1.0.

The various factors favouring complete conversion of the starting material (*i.e.*, increased time, temperature, and pH, and use of DMF, TEA buffer, and a 3–12-fold excess of FDNB) were, to a considerable extent, associated with the formation of by-products. It was considered that the lower reactivity in ethanol and formation of 1-ethoxy-2,4-dinitrobenzene were preferable to the increased chance of by-product formation associated with other solvents. The method finally recommended employs ethanolic FDNB in 0.5M sodium carbonate–hydrogencarbonate buffer (pH 9.0) at room temperature for 2 h with vigorous stirring. A 33% excess of FDNB was used when it was desired merely to convert a compound into its Dnp-derivative for qualitative identification or for preparative purposes where a reasonable, but not quantitative, yield was required without too much contamination by dinitrophenol, 1-ethoxy-2,4-dinitrobenzene, and unreacted FDNB. For quantitative work, up to a 12-fold molar excess of FDNB was required. These reaction conditions represent a fine balance between incomplete derivatisation at pH 8.5 (some $MurH_2$ remained unreacted even after 5 h at pH 8.5 with a 12-fold molar excess of FDNB) and increased formation of by-products at pH 9.5 (after 2 h, $MurH_2$ gave a noticeably more intense spot, R_F 0.11). With care, the formation of by-products could be almost completely avoided at pH 9.0, except in the case of Dnp-Mur.

Although reasonably good yields, with minimal levels of by-products, could be obtained by careful monitoring of preparative experiments by t.l.c., it is unrealistic to expect reproducible, stoichiometric production of the Dnp-derivatives when a series of small samples is derivatised for analytical purposes. The narrow pH-range for optimum derivatisation is consistent with the findings of Haas and Weigerding[2] that the yield of the desired product is very sensitive to the proportions of base and amino sugar. This finding casts doubt on the value of Dnp-derivatives of amino sugars for quantitative microanalysis unless the method is combined with isotope dilution. A similar approach was successfully applied to amino acid analysis following derivatisation by a reaction yielding by-products[11]. In agreement with Leskowitz and Kabat[4], aminodeoxyalditols are more suitable for analysis than the parent amino sugars, since the former compounds never form unknown by-products during

derivatisation unless TEA or DMF is present or the pH is too high. Moreover, they are more stable to heating in mildly alkaline conditions (see below).

Although t.l.c. has been used[12] to identify Dnp-Mur, the occurrence of by-products was not mentioned. Fortuitously, only solvent *C* had been used in the earlier work; in the present experiments, solvent *C* produced only a single, rather elongated spot with a sample of Dnp-Mur that clearly contained the two major by-products when run in solvent *B*. The muramic acid used was analytically satisfactory, gave only a single, detectable spot (R_F 0.54) when examined in solvent *A*, and was free from isomuramic acid (R_F 0.47). Moreover, authentic isomuramic acid gave a different set of Dnp-derivatives, which moved slightly faster in solvent *B* (see Table I) than those of muramic acid.

Further evidence which supports the view that the 3 spots obtained with Dnp-Mur arise by derivatisation of a single amino compound came from preparative t.l.c. experiments. The three bands with R_F 0.26, 0.36, and 0.44 were unstable. When extracted and re-run in the same system, the first component gave all three spots. The other two bands produced even faster-running spots together with a spot R_F 0.26, which suggests that, to some extent, the three major components are interconvertible. Furthermore, when reaction mixtures were sampled over several hours, the results suggested precursor–product relationships. Especially with DMF present, the desired Dnp-products started to decline, slower-moving by-products became more prominent, and, eventually, faster-moving by-products appeared.

The presence of FDNB was not essential for by-product formation from purified Dnp-amino sugars. When heated at 70° for 5 min with 1:1 TEA-CO_2 buffer (pH 8.0)–DMF or in 0.15M Na_2CO_3, Dnp-Mur produced faster-moving spots much more readily than Dnp-GlcN. In confirmation of the more robust nature of the corresponding alcohols, no by-products were detectable after similar treatment.

Formation of by-products from the least reactive compound, Dnp-$GlcNH_2$, occurred only with FDNB present. It is suggested that, as with 1-ethoxy-2,4-dinitrobenzene production, etherification of hydroxyl groups is involved. Both processes occurred to a very small extent unless TEA or DMF was present. Tertiary bases are known to facilitate etherification considerably[13], perhaps *via* a quaternary amine, transition-state intermediate arising from FDNB[14]. Alditol hydroxyl groups can be completely substituted by TEA-catalysis in anhydrous DMF[15,16], in which alkoxide anions are relatively poorly solvated and highly reactive.

The formation of by-products can also occur under acid conditions. Dnp-$MurH_2$ was converted into a product having R_F 0.45 (solvent *B*) by treatment with a strongly acidic ion-exchange resin [Amberlite CG-120(H^+) form] at room temperature, or in high yield by heating in 50% acetic acid at 100° for 5 min. Unlike the starting material, the product was immobile when subjected to electrophoresis at pH 6.5, suggesting that lactonisation might have occurred.

Thus, amino sugars and their alcohols can be converted into Dnp-derivatives without undue formation of by-products if conditions are carefully controlled. It would be wise to employ equal caution in extending this label to other amino sugars

(such as the neuraminic acids) or in using other types of nucleophilic reaction for labelling amino sugars. Even though the detailed chemistry of the formation of by-products remains to be worked-out, the conditions are now probably sufficiently well-defined for Dnp-derivatives to be used reliably for a variety of analytical purposes. These could include determination of individual hexosamines in acid hydrolysates, the specific activities of precursors and products in biosynthetic experiments, and glycan chain-lengths from the proportion of aminodeoxyhexitols following reduction with borohydride.

Any separation process used in quantification should be chosen with care. Considerable losses can occur, especially if attempts are made to scale-down the process[1,4,8], and probably account for some of the relatively low yields of Dnp-GlcN reported in the literature. Silica gel, particularly when activated, tends to cause fading of Dnp-derivatives during separation or if stored dark in contact with the support medium. Systems have recently been developed[17] for the separation of Dnp-amino sugars and their alcohols by polyamide t.l.c., a milder but more expensive separation medium that is superior to silica gel when samples must be recovered for liquid scintillation counting[18].

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