# Trehalose Covalently Conjugated to Bovine Serum Albumin

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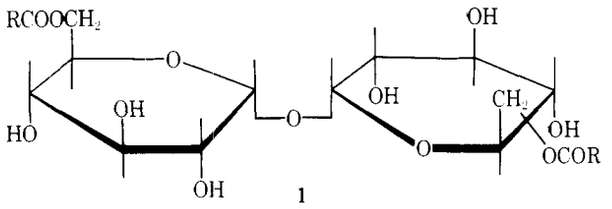
Trehalose has been covalently bonded to bovine serum albumin through both primary hydroxyl groups at the disaccharide 6 and 6′ positions, and also singly bonded through one of these positions.

The family of bacterial liposaccharides collectively called "cord factors" or 6,6′-dimycolyltrehalose (**1**)[1] exhibits a variety




**1**

of significant physiological, biochemical, and immunological properties.[2,3] For several of these activities, the presence of the trehalose portion substituted at the 6,6′ positions has been found to be either necessary or optimal.[3-5] From these observations and from the fact that cord factor simply mixed with a protein before administration is more active than cord factor alone,[5,6] we were led to consider the effect of *covalently* linking trehalose through its 6- and 6′-hydroxyl groups to a protein carrier. The present paper describes the first example of such a conjugated protein (**11**) in which bovine serum albumin is taken as the protein and *p*-aminobenzoic acid units serve conveniently as connecting links. Further, to allow determination of the effect of only one link between disaccharide and protein, we synthesized the same kind of conjugated protein attached to trehalose through *p*-aminobenzoic acid but only at the disaccharide 6 position (cf. **17**).

The synthesis plan relied either on a useful difference in reactivity between the primary and secondary hydroxyl groups of trehalose or on suitably blocked molecules. Although the literature describes several selective reactions favoring the primary positions,[7] our own attempts at direct esterifications at the trehalose 6,6′ positions gave only unsatisfactory mixtures.[8] An attractive sequence starting with the well-characterized 6,6′-ditrityltrehalose[9] proved disappointing when it was found that the open 2,3,4,2′,3′,4′-hydroxyl groups resisted complete alkylation with methoxymethyl halide.[10] We then turned to octa(trimethylsilyl)trehalose (**3**), which could be prepared in good yield by treating trehalose with trimethylsilyl chloride in dry pyridine. This octasilylated derivative could be selectively desilylated to hexasilylated trehalose **4** having both primary hydroxyl groups exposed, or to the heptasilylated trehalose **12** with only one primary hydroxyl exposed.[11] The presence of two hydroxyl groups in **4** was confirmed by forming diacetyl derivative **5.**

Evidence that the exposed hydroxyl groups correspond to the two primary hydroxyl groups at the 6,6′ positions was provided by the NMR data. Thus, in hexasilylated trehalose **4,** the signal at $\delta$ 2.1 ppm for the hydroxyl groups (lost on addition of deuterated water) appears as a two-proton triplet, a feature consistent only with two equivalent OH's both adjacent to vicinal methylene groups, as in assigned structure **4.** Furthermore, in the derived diacetate **5,** the $\delta$ 3.6 ppm signal for the 6,6′-methylene groups integrates to four protons, which value would not be obtained if one or both of the acetyl groups were attached anywhere other than to the two primary hydroxyls.

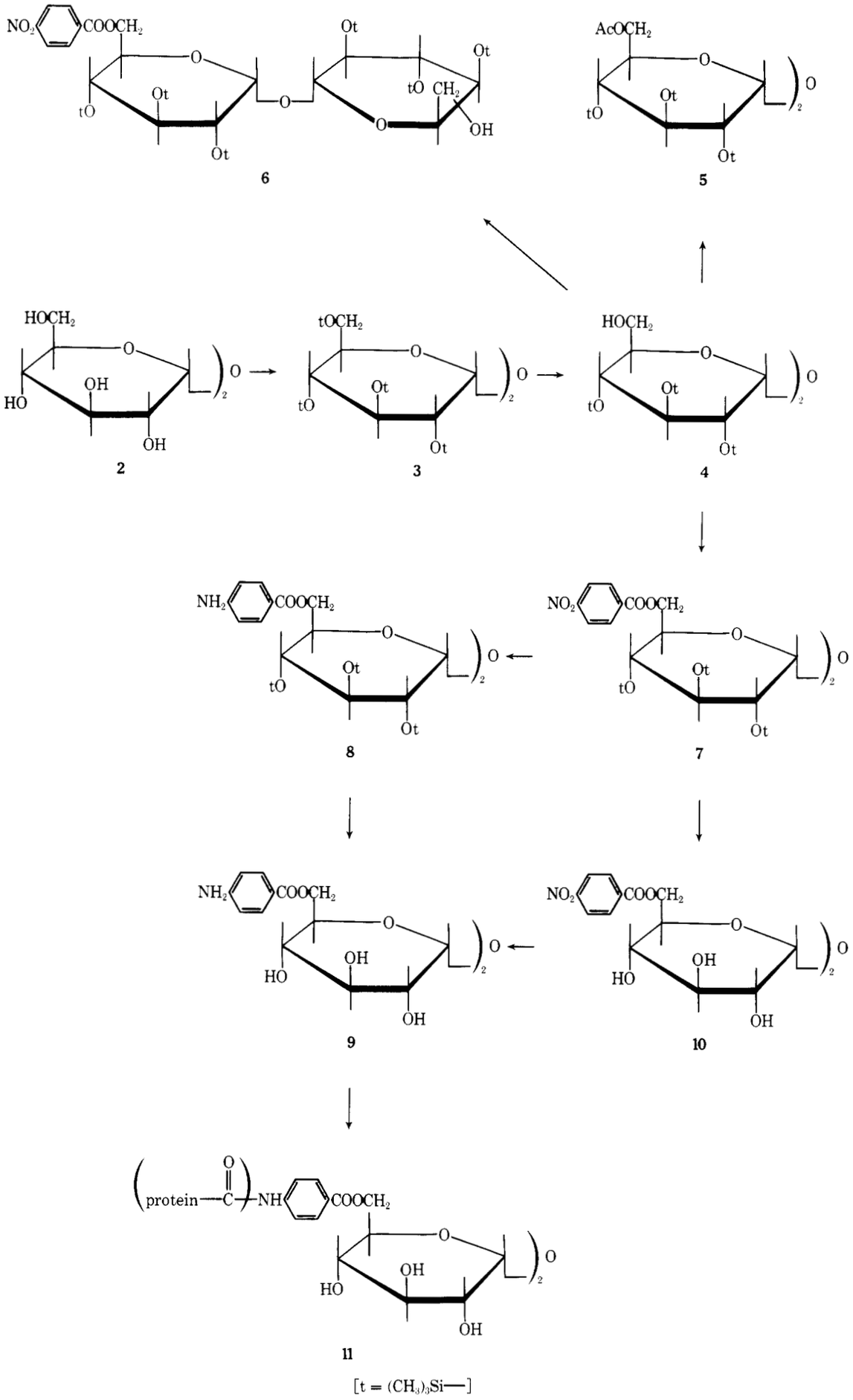
In order to provide the bridging *p*-aminobenzoate sections, the primary hydroxyl groups at the 6,6′ positions were first esterified with *p*-nitrobenzoyl groups. Taking *p*-nitrobenzoyl chloride as reagent, the only pure product isolated in low yield was monoester **6.** The possibility that chloride ion, by displacing one or more trimethylsilyl blocking groups, led to undesirable products was supported by the observation that persilylated trehalose **3** dissolved in pyridine containing pyridinium hydrochloride was completely converted to a mixture after standing at room temperature for 1 day.[12] Esterification with the mixed anhydride of *p*-nitrobenzoic and benzenesulfonic acids[13] furnished the desired di(*p*-nitrobenzoyl) ester **7** plus small amounts of monoester **6.** Catalytic hydrogenation reduced the nitro groups of **7** and gave hexasilylated 6,6′-di(*p*-aminobenzoyl)trehalose (**8**), which was easily transformed to 6,6′-di(*p*-aminobenzoyl)trehalose (**9**). The alternate course of first removing the protecting groups from the di(*p*-nitrobenzoate) **7** and then reducing the resulting 6,6′-di(*p*-nitrobenzoyl)trehalose (**10**) offered little advantage.

To arrive at the conjugated protein, a water-soluble carbodiimide was used to form amide links between side-chain carboxyl groups of bovine serum albumin and the amino groups of diamine **9.**[14] After the coupling, dialysis and lyophilization yielded the finished product **11,** which by assay was shown to carry an average of seven prosthetic groups per molecule of protein.

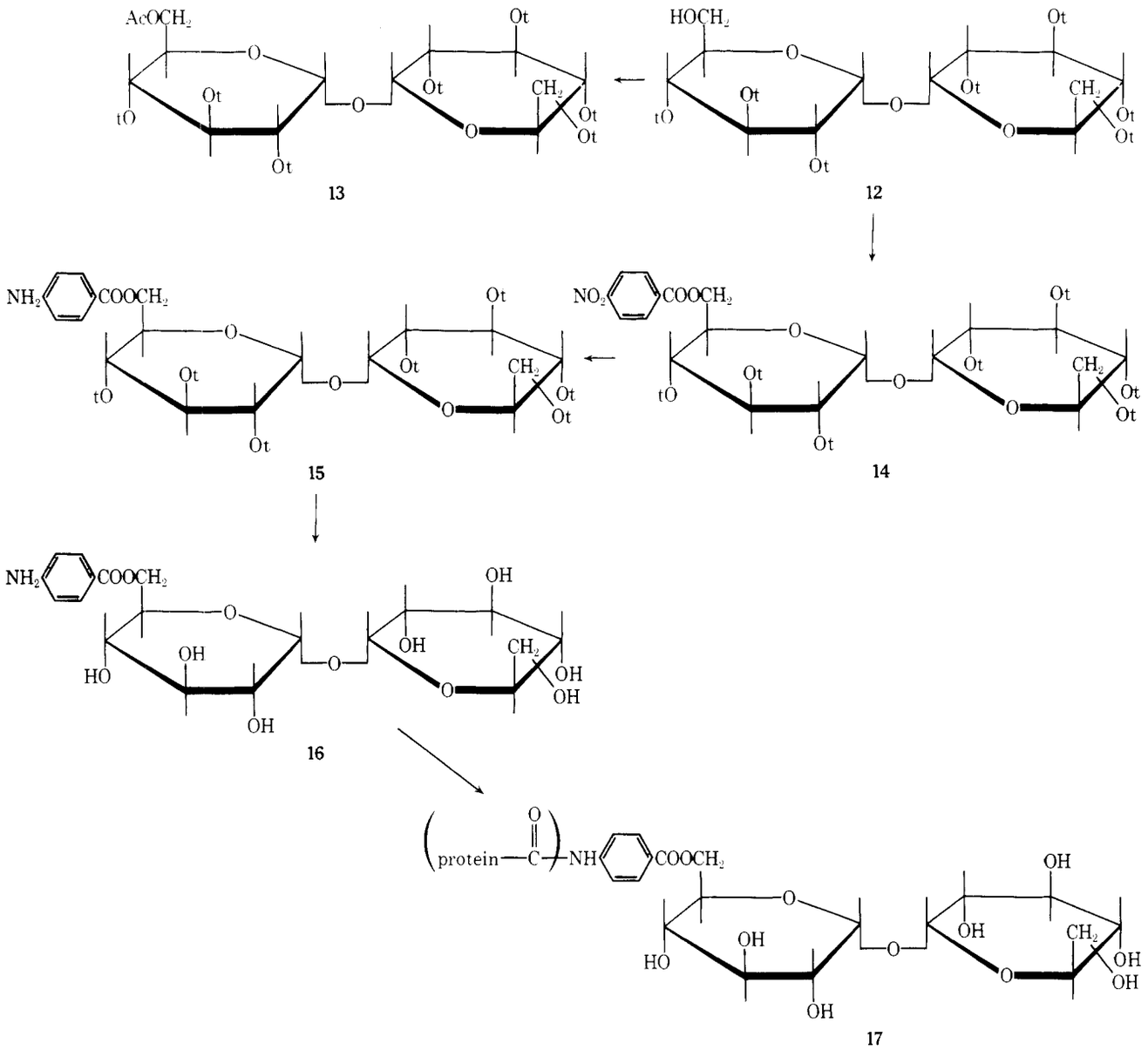
Under properly controlled conditions, the persilylated trehalose **3** could be converted to 2,3,4,2′,3′,4′,6′-hepta(trimethylsilyl)trehalose (**12**). Although the conversion was modest (40%), the yield corrected for recovered starting material **3** and hexasilylated derivative **4** was over 90%. The presence of an open hydroxyl group in **12** was confirmed by acetylation to monoacetyl derivative **13.** The lone exposed hydroxyl group in heptasilylated compound **12** was shown to be the primary group at the 6 (or 6′) position, since the derived nitrobenzoyl derivative **14** obtained by acylating the heptasilylated derivative **12** proved to be identical with the compound obtained by silylating the mononitrobenzoyl from the 6,6′-dihydroxy hexasilylated trehalose **6.** The assignment for **12** was further supported by controlled hydrolysis of the heptasilylated derivative **12,** which led to the 6,6′-dihydroxy compound **4.**

The procedures for attaching the *p*-nitrobenzoyl group to form **14,** hydrogenation to 6-(*p*-aminobenzoyl)hepta(trimethylsilyl)trehalose (**15**), and then removing the masking groups to give 6-(*p*-aminobenzoyl)trehalose (**16**) were similar to those employed in the bis series, as was the final coupling process that produced the conjugated protein **17.** This material assayed for four to five prosthetic groups per molecule of protein.

Samples of the two conjugated proteins **11** and **17** have been distributed to several laboratories for biological assays. Our further plans call for synthesis of analogous conjugated proteins, varying the protein as well as the nature and length of the connections between protein and trehalose.



[t = $(CH_3)_3Si$—]



## Experimental Section

**General.** Temperatures are uncorrected. The nuclear magnetic resonance curves were determined at 60 MHz, with the chloroform peak (δ 7.42 ppm) used as an internal reference in place of tetramethylsilane when it was necessary to avoid interference with the signals from the trimethylsilyl groups. Sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate served as an internal reference in the aqueous systems. The reactions were run under nitrogen gas that had been passed through a column of anhydrous calcium sulfate (Drierite). When anhydrous pyridine was used as solvent, it was distilled from calcium hydride and then stored over a molecular sieve (3 Å) under nitrogen. Almost all starting reagents were distilled just before use. The petroleum ether used here boiled at 38–40 °C. We relied on commercial 5 × 20 cm silica plates (0.25 mm thickness) for thin layer chromatography, with spots brought out by spraying the plate with 5% concentrated sulfuric acid in ethanol and then heating. Analysis for elements were reported by Galbraith Laboratories, Inc., Knoxville, Tenn. Molecular weight determinations were performed by mass spectrometry on an AEI MS-9 instrument.

**Octa(trimethylsilyl)trehalose (3).** Anhydrous trehalose **2**[15] (6.8 g, 0.020 mol) was dissolved in dry pyridine (200 ml), and to this solution at room temperature was added trimethylsilyl chloride (21.8 g, 0.20 mol) over a 0.5-h period. After stirring at room temperature for 1 day, the mixture was refluxed for 2 h. Solvent was removed by distillation at reduced pressures at temperatures below 50 °C. The residue, treated with 200 ml of dry petroleum ether, was filtered. Distillation of the concentrated filtrate in a wide-bore short-path still afforded 12 g (66%) of persilylated trehalose **3**, bp 195–200 °C (0.1 mm), which showed one spot on TLC, $R_f$ 0.86 (ether–petroleum ether, 1:15), and melted at 75–78 °C. GLC through 3% SE-30 supported on Chromosorb at a column temperature of ca. 295 °C gave one peak with retention time 21 min; ir, no absorption around 3500 $cm^{-1}$; NMR ($CDCl_3$) δ 4.9 (d, $J$ = 3 Hz, 2, H-1,1′), 4.1–3.2 (m, 12, all other HCO's), 0.2 ppm [s, 72, $(CH_3)_3Si$'s].

Anal. Calcd for $C_{36}H_{86}O_{11}Si_8$: C, 47.06; H, 9.37; Si, 24.40. Found: C, 47.22; H, 9.28; Si, 24.19.

**Hexa(trimethylsilyl)trehalose (4) with Unsubstituted 6- and 6′-Hydroxy Groups.** Water (0.9 g, 50 mmol) was added to an 8–10 °C solution of persilylated trehalose **3** (4.6 g, 5.0 mmol) in 100 ml of pyridine. Glacial acetic acid (0.6 g, 10 mmol) was then introduced, and the cold solution was stirred under nitrogen for 5 h. At this time no TLC spot corresponding to starting material could be detected. The reaction mixture was poured over crushed ice and water (400 ml), and the two-phase system was extracted with several portions of petroleum ether. After the combined extract was washed several times with cold water, the organic layer was dried and concentrated at room remperature under water-pump vacuum. The resulting solid was placed on a Florisil column and chromatographed, using as eluents 400 ml of ether–petroleum ether (1:5) (the first 150 ml was discarded) and then ether–petroleum ether (1:3). Complete removal of all volatiles under reduced pressure at room temperature left 3.0 g (76%) of the desired product **4**: mp 116–118 °C; TLC showed one spot, $R_f$ 0.4, using ether–petroleum ether (1:1); ir ($CHCl_3$) 3600 $cm^{-1}$; NMR ($CDCl_3$) δ 4.8 (d, $J$ = 3 Hz, 2, H-1,1′),4.1–3.2 (m, 12, all other HCO's), 2.1 (broad t, $J$ = 3 Hz, 2, 2 OH's), 0.15–0.13 ppm (m, 54, all $CH_3Si$). When a drop of deuterated water was added, the 2.1-ppm signal disappeared in favor of one at 4.7 ppm.

Anal. Calcd for $C_{30}H_{70}O_{11}Si_6$: C, 46.51; H, 9.04; Si, 21.71; mol wt, 774. Found: C, 46.62; H, 9.03; Si, 21.64, mol wt, 774 (30 eV, with injection port at ca. 210 °C).[16]

**Hepta(trimethylsilyl)trehalose (12) with Unsubstituted 6-Hydroxy Group.** Pyridine (150 ml) was injected onto 4.6 g (5.0 mmol) of persilyltrehalose **3** in a three-necked flask stoppered with rubber septums. After the mixture was cooled to 5 °C, water (0.45 g, 25 mmol) followed by acetic acid (0.3 g, 5 mmol) was introduced, and the solution was stirred for 2 h. When TLC results (ether–petroleum ether, 1:15) showed that the intensity of the developing spot at $R_f$ 0.65 had peaked, the mixture was quenched on ice–water (500 ml) and extracted with petroleum ether. The extract was washed with several portions of cold water, dried without delay ($Na_2SO_4$), and concentrated under reduced pressures at temperatures no higher than ambient. The viscous residue, which contained three components by TLC, was chromatographed through a 30 × 1.3 cm Florisil column using the sequence of solvents, petroleum ether (300 ml), 1:15 ether–petroleum ether (150–200 ml), and ether (50 ml). Unchanged persilylated trehalose (mp 75–78 °C, $R_f$ 0.86) emerged first with the petroleum ether (1.7 g, 37% recovery); the desired monohydroxy product **12** came next with the mixed solvent, followed finally by 0.5 g (19%) of the dihydroxy compound **4** (mp 116–118 °C, $R_f$ 0.28). Monohydroxy derivative **12** was obtained as a colorless, very viscous liquid (1.05 g, 39%), which slowly hardened when allowed to stand at room temperature under vacuum. It showed only one TLC spot with $R_f$ 0.65 using 1:15 ether–petroleum ether or 0.75 with 1:1 ether–petroleum ether: ir ($CHCl_3$) 3600 $cm^{-1}$; NMR ($CDCl_3$) $\delta$ 4.95 (complex t, $J$ = 3 Hz, 2, H-1, 1′), 4.2–3.3 (m, 12, all HCO), 1.85 ppm (broad triplet, 1, OH). With ordinary chloroform as internal standard, the trimethylsilyl group signals were seen at $\delta$ 0.3 ppm (m, 62 H as compared to the required 63). The OH peak disappeared when $D_2O$ was added.

Anal. Calcd for $C_{33}H_{78}O_{11}Si_7$: C, 46.81; H, 9.22; Si, 23.17; mol wt, 846. Found: C, 46.94; H, 9.06; Si, 23.30; mol wt, 846 (mass spectrometric value obtained at 30 eV with injection temperature at ca. 205 °C).

When heptasilylated trehalose **12** (0.42 g) in 15 ml of pyridine was stirred for 4 h at 5–10 °C with 0.09 g of water and 0.10 g of glacial acetic acid, and the reaction mixture was processed essentially as before, the product was the hexasilylated derivative **4** (62%), mp 116–118 °C, one spot on TLC with $R_f$ 0.4 (1:1 ether–petroleum ether).

**6,6′-Diacetylhexa(trimethylsilyl)trehalose (5).** With moisture rigorously excluded, 1 ml of acetic anhydride was injected into a stirred mixture of dihydroxy compound **4** (0.19 g, 0.24 mmol) and dry pyridine (10 ml) at 10–15 °C. After 40 h of stirring at room temperature, the mixture, which according to TLC no longer contained starting material, was poured into ice–water (150 ml). Filtering the precipitate and then drying gave crude diacetylated product **5**. Chromatography through a 12 × 0.5 cm column of Florisil with 30 ml of ether–petroleum ether (1:5) as solvent afforded 0.13 g (59%) of 6,6′-diacetylhexa(trimethylsilyl)trehalose (**5**): mp 158–160 °C; one spot on TLC with ether–petroleum ether (1:7) solvent, $R_f$ 0.71; NMR ($CDCl_3$) $\delta$ 5.05 (d, $J$ = 3 Hz, 2, H-1,1′), 4.4–3.85 (m, 8), 3.6 (m, 4, 2 $CH_2O$), 2.15 (s, 6, 2 $CH_3COO$), 0.15 ppm [m, 51 as compared to 54 required, $(CH_3)_3Si$'s].

Anal. Calcd for $C_{34}H_{74}O_{13}Si_6$: C, 47.55, H, 8.62; Si, 19.58; mol wt, 858. Found: C, 47.64; H, 8.65; Si, 19.81; mass spectral mol wt (70 eV with injection port at ca. 215 °C), 858, as a low-intensity peak. A very minor peak was also seen at $m/e$ 934.

**6,6′-Di(*p*-nitrobenzoyl)hexa(trimethylsilyl)trehalose (7).** Benzenesulfonyl chloride (1.4 g, 8.0 mmol) in one portion was injected into a 0–5 °C stirred solution of sublimed *p*-nitrobenzoic acid (0.67 g, 4.0 mmol) dissolved in 100 ml of dry pyridine. After the mixture had been stirred for about 5 min, a solution of dihydroxy derivative **4** (1.6 g, 2.0 mmol) in pyridine (10 ml) was added over a 1-min period. The stirred mixture was held at 0–5 °C for 24 h before it was poured into 300 ml of ice–water. Filtration gave the crude product, which was dried for a short time and then chromatographed through a 30 × 0.5 cm column of Florisil with ether–petroleum ether (1:15) as solvent.

Mono-*p*-nitrobenzoate **6** (0.13 g, 7%), coming out in the first 200 ml of eluate, showed mp 63–65 °C and $R_f$ 0.5 (ether–petroleum ether, 1:5).

The next 300 ml removed the desired di(*p*-nitrobenzoyl)hexa(trimethylsilyl)trehalose (0.84 g, 39%), homogeneous by TLC ($R_f$ 0.21), but crystallized only with difficulty from petroleum ether: mp 87–91 °C; ir ($CHCl_3$) 1750 $cm^{-1}$ with no OH absorption at 3500 $cm^{-1}$; NMR ($CDCl_3$) $\delta$ 8.25 (s, 8, ArH's), 4.98 (d, $J$ = 3 Hz, 2, H-1,1′), 4.5–3.3 (m, 13 as against 12 required for HCO's), 0.15 ppm [m, 54, $(CH_3)_3Si$'s].

Anal. Calcd for $C_{44}H_{76}N_2O_{17}Si_6$: C, 49.25; H, 7.09; N, 2.61; Si, 15.67; mol wt, 1072. Found: C, 49.40; H, 6.93; N, 2.62; Si, 15.77; mass spectral mol wt (30 eV at injection temperature ca. 215 °C), 1072.

As proved by TLC monitoring, esterification attempts using separately prepared *p*-nitrobenzoic anhydride[13] in hot pyridine failed, the starting material persisting unchanged.

**6-(*p*-Nitrobenzoyl)-2,3,4,2′,3′,4′-hexa(trimethylsilyl)trehalose (6).** *p*-Nitrobenzoyl chloride (0.48 g, 2.6 mmol) in 10 ml of pyridine was injected through a septum into pyridine (100 ml) under a nitrogen atmosphere. This was followed by hexasilylated trehalose **4** (1.0 g, 1.3 mmol) in 10 ml of pyridine, and the stirred mixture was refluxed for 1 h. Complete removal of volatiles under reduced pressures and at temperatures no higher than 50 °C left a dry residue, a solution of which in dry petroleum ether was filtered to remove insoluble pyridinium hydrochloride. After solvent was removed, the mixture remaining was chromatographed through Florisil with 1:10 ether–petroleum ether as solvent (ca. 100 ml). Those fractions showing only a single TLC spot at $R_f$ 0.5 (ether–petroleum ether, 1:5) were combined and stripped of volatiles. (*p*-Nitrobenzoyl) hexasilylated trehalose **6** (0.13 g, 12%) was obtained in this way with mp 63–65 °C. Crystallization from petroleum ether, although possible, was not satisfactory: ir ($CHCl_3$) 3500, 1750 $cm^{-1}$; NMR ($CDCl_3$) $\delta$ 8.25 (s, 4, ArH's), 5.0 (t, $J$ = 2 Hz, 2, 2 H-1's), 4.9–3.3 (m, 12, remaining OCH's), 0.15 ppm [m, 54, $(CH_3)_3Si$]. A signal at $\delta$ 1.25 ppm was attributed to the hydroxyl group, although the integration was too small for one proton.

Anal. Calcd for $C_{37}H_{73}NO_{14}Si_6$: C, 48.10; H, 7.90; N, 1.51; Si, 18.20; mol wt, 923. Found: C, 47.99; H, 7.94; N, 1.48; Si, 18.13; mass spectral mol wt (30 eV with $T$ = 215 °C), 923.

When a sample of this material **6** was exposed to the action of trimethylsilyl chloride in pyridine and the course of the reaction monitored by TLC, a progressively darker spot developed corresponding exactly in its $R_f$ value with that of 6-(*p*-nitrobenzoyl)hepta(trimethylsilyl)trehalose (**14**).

Carrying out the *p*-nitrobenzoyl chloride esterification at room temperature instead of at the boiling point of pyridine gave no reaction at all.

**6,6′-Di(*p*-aminobenzoyl)hexa(trimethylsilyl)trehalose (8).** After 25 mg of 5% palladium on carbon had been stirred for a few minutes with 50 ml of ethyl acetate under a small positive pressure of hydrogen, a 0.10-g (0.090 mmol) sample of dinitro compound **7** already in the hydrogenation vessel was tipped into the catalyst mixture. Stirring for 4 h resulted in the uptake of the calculated amount of hydrogen, after which time no more hydrogen was absorbed. Filtration followed by evaporation of the filtrate under reduced pressure at temperatures no higher than 40 °C left a solid, mp 226–227 °C, which was chromatographed on a 20 × 1 cm column of Florisil using ether–petroleum ether (7:3) as solvent. The eluted, essentially pure product (71 mg, 82%) was crystallized with difficulty from ether–petroleum ether (1:1) to give needlelike crystals of 6,6′-di(aminobenzoyl)hexa(trimethylsilyl)trehalose (**8**): mp 226–227 °C; homogeneous according to TLC with $R_f$ 0.5 (ether solvent); ir (KBr) 3500, 3400, 1720 $cm^{-1}$; NMR ($CDCl_3$) $\delta$ 7.8 (d, 4, 4 H ortho to carbonyls), 6.6 (d, 4, 4 H ortho to $NH_2$ groups), 4.95 (d, $J$ = 3 Hz, 2, H-1,1′), 4.7–3.3 (m, 16, remaining HC and HN's), 0.15 ppm [m, 53 as compared with the calculated 54, $(CH_3)_3Si$'s].

Anal. Calcd for $C_{44}H_{80}N_2O_{13}Si_6$: C, 52.17; H, 7.90; N, 2.76; Si, 16.60, mol wt, 1012. Found: C, 51.95; H, 8.14; N, 2.69; Si, 16.71, mass spectral mol wt (30 eV at 275 °C), 1012.

**6,6′-Di(*p*-nitrobenzoyl)trehalose (10).** The masking trimethylsilyl groups were removed from 0.27 g (0.25 mmol) of 6,6′-di(*p*-nitrobenzoyl)hexa(trimethylsilyl)trehalose (**7**) by refluxing the material for 2.5 h with 25 ml of methanol and 5 ml of water plus 1 drop of acetic acid. The solvent-free product, 6,6′-di(*p*-nitrobenzoyl)trehalose (**10**), was isolated in near quantitative yield. TLC using chloroform–methanol (2:1) as solvent developed only a single spot, $R_f$ 0.21. Crystallization from methanol gave 0.12 g of product with mp 149–151 °C (previous softening); ir (KBr) 3400, 1725 $cm^{-1}$; NMR (in deuterated dimethyl sulfoxide–water–acetone mixed solvent) $\delta$ 8.4 (d of d, 8, ArH's), 5.1 (broad d, $J$ = 2.5 Hz, 2, H-1,1′), 4.25 (s, HOD), 4.8–3.2 ppm (m, 12, remaining H's).

Anal. Calcd for $C_{26}H_{28}N_2O_{17}\cdot 2H_2O$: C, 46.15; H, 4.73; N, 4.14. Found, C, 45.91; H, 4.82; N, 4.00.

**6,6′-Di(aminobenzoyl)trehalose (9). A.** A slightly turbid solution of 0.63 g (0.59 mmol) of 6,6′-di(*p*-aminobenzoyl) hexasilylated trehalose **8** and 1 drop of acetic acid in methanol (100 ml) plus water (15 ml) was refluxed for 1.5 h, at which point starting material could no longer be detected by TLC. The residue remaining after solvent was removed (vacuum at 50 °C) was dissolved in water, and the mixture was filtered. Complete evaporation of the water at 50 °C under reduced pressure left 0.29 g (77%) of slightly hygroscopic di(aminobenzoyl)trehalose (**9**): mp 115–119 °C with previous softening; one spot ($R_f$ 0.5) on TLC with methanol–chloroform (1:1); NMR ($D_2O$)

δ 7.8 (d of d, 4 + 4, 8 ArH's), 5.2 (broad m, 2, H-1,1'), 4.85 (s, HOD), 4.65–3.4 ppm (m, 14 as compared to 12 required, all remaining HC's).

Anal. Calcd for $C_{26}H_{32}N_2O_{13}\cdot H_2O$: C, 52.17; H, 5.68; N, 4.68. Found: C, 51.98; H, 5.54; N, 4.55.

**B.** 6,6'-Di(*p*-nitrobenzoyl)trehalose (**10**, 64 mg, 0.10 mmol) in 100 ml of 1:1 methanol–ethyl acetate in the presence of 5 mg of 5% palladium on carbon was stirred under an atmosphere of hydrogen for 7 h. Removal of catalyst and solvent left 37 mg (64%) of 6,6'-di(aminobenzoyl)trehalose (**9**), mp 115–119 °C (softening). Repeated recrystallizations from methanol did not change the melting point. Although some minor spots appeared on a TLC plate (methanol–chloroform, 1:1), the single predominant spot showed an $R_f$ value mtching that of the material described in part A; a mixture of the two diamines **9** showed mp 116–121 °C (preliminary softening).

**6-Acetylhepta(trimethylsilyl)trehalose (13).** Acetic anhydride (1 ml) was injected into a stirred, 0–5 °C solution of 0.85 g (1.0 mmol) of hepta(trimethylsilyl)trehalose **12** in 25 ml of pyridine, and the mixture was stirred at room temperature for 48 h. Pouring the mixture into 200 ml of ice–water precipitated a solid, which was collected on the funnel, dried, and chromatographed through Florisil using ether–petroleum ether (1:30) as eluting solvent. Homogeneous 6-acetylhepta(trimethylsilyl)trehalose (**13**) ($R_f$ 0.68 with 1:15 ether–petroleum ether) was obtained in this way as a solid (0.72 g, 80%): mp 82–85 °C; NMR ($CDCl_3$) δ 4.98 (t, $J$ = 3 Hz, 2, H-1,1'), 4.4–3.3 (m, 12, remaining OCH's), 2.1 (s, 3, $CH_3CO$), 0.15 ppm [m, 59 with 63 required for **13**, $(CH_3)_3Si$'s].

Anal. Calcd for $C_{35}H_{80}O_{12}Si_7$: C, 47.29; H, 9.00; Si, 22.07; mol wt, 888. Found: C, 47.44; H, 9.07; Si, 22.09; mass spectral mol wt, 888.

**6-(*p*-Nitrobenzoyl)hepta(trimethylsilyl)trehalose (14).** The procedure developed for the di(*p*-nitrobenzoyl) derivative **7** was applied in its essentials to the reactants hepta(trimethylsilyl)trehalose **12** (3.4 g, 4.0 mmol), *p*-nitrobenzoic acid (1.0 g, 6.0 mmol), and benzenesulfonyl chloride (2.1 g, 12 mmol) in pyridine (150 ml). The crude oily product was taken up in petroleum ether (300 ml), and the solution was washed with water, dried, and stripped of solvent without heating. The residue was chromatographed as before. After the first 150 ml of eluate was rejected (1:15 ether–petroleum ether), the next 300 ml was collected and stripped of volatiles to leave 1.3 g (32%) of homogeneous 6-(*p*-nitrobenzoyl)hepta(trimethylsilyl)trehalose (**14**): mp 60–63 °C; $R_f$ 0.54 on TLC with ether–petroleum ether (1:15) as developing solvent; ir (KBr) 1750 $cm^{-1}$; NMR ($CDCl_3$) δ 8.4 (s, 4, ArH's), 5.2 (broad t, $J$ = 2.5 Hz, 2, H-1,1'), 4.9–3.3 (m, 12, remaining OCH's), 0.15 ppm [m, 62 vs. 63 required, $(CH_3)_3Si$'s].

Anal. Calcd for $C_{40}H_{81}NO_{14}Si_7$: C, 48.24; H, 8.14; N, 1.40; Si, 19.65; mol wt, 995. Found: C, 48.44; H, 8.29; N, 1.46; Si, 19.81; mass spectral mol wt, 995.

**6-(*p*-Aminobenzoyl)hepta(trimethylsilyl)trehalose (15).** Hydrogenation of 0.50 g (0.5 mmol) of the mono(*p*-nitrobenzoyl) compound **14** in 40 ml of ethyl acetate was conducted essentially the same way as described for the di(*p*-nitrobenzoyl) derivative **7**. After 100 min of stirring and the absorption of 120% of the calculated volume of hydrogen, no further hydrogen uptake was noted. Evaporation of the ethyl acetate afforded 0.47 (98%) of solid with mp 211–213 °C that was taken as aminobenzoylhepta(trimethylsilyl)trehalose **15**. Recrystallization of this material (one spot on TLC with $R_f$ 0.57 using ether–petroleum ether, 8:2) from 1:1 ether–petroleum ether failed to give well-formed crystals, and did not change the melting point: ir (KBr) 3400, 3325, and 1725 $cm^{-1}$; NMR ($CDCl_3$ with $CHCl_3$ as internal reference with δ 7.42 ppm) δ 7.89 (d, 2, H's ortho to amino), 6.65 (d, 2, H's ortho to ester carbonyl), 4.95 (distorted t, 2, H-1,1'), 4.87–3.15 (m, 14, remaining OCH's), 0.13 [m, 63, $(CH_3)_3Si$].

Anal. Calcd for $C_{40}H_{83}NO_{12}Si_7$: C, 49.74; H, 8.60; N, 1.45; Si, 20.31; mol wt, 965. Found: C, 49.76; H, 8.74; N, 1.42; Si, 20.31; mass spectral mol wt, 965.

**6-(*p*-Aminobenzoyl)trehalose (16).** The trimethylsilyl protecting groups were removed from 0.29 g (0.30 mmol) of *p*-aminobenzoyl heptasilylated trehalose **15** dissolved in 25 ml of methanol by following the directions for the di(*p*-aminobenzoyl) analogue **8**. The reaction was complete in 2 h. The dry product **16**, homogeneous by TLC ($R_f$ 0.38 with 4:1 methanol–chloroform) and obtained in 95% yield, was crystallized from methanol to give 6-(*p*-aminobenzoyl)trehalose (**16**): mp 148–151 °C; ir (KBr) 1725 $cm^{-1}$; NMR ($D_2O$) δ 7.95 (d, 2, 2 H ortho to ester), 6.79 (d, 2, 2 H ortho to amino), 5.28 (t or overlapping d, $J$ = 2 Hz, 2, H-1,1'), 4.85 (s, HOD), 4.7–3.4 ppm (m, 12, all other CH's).

Anal. Calcd for $C_{19}H_{27}NO_{12}\cdot 1.5H_2O$: C, 46.72; H, 6.14; N, 2.86. Found: C, 46.81; H, 6.53; N, 2.67.

**Attaching the Prosthetic Groups 9 and 16 to Protein.** A solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.5 g, 1.2 mmol) in 1.5 ml of water was added to a solution of 100 mg of either the diamino compound **9** (0.17 mmol) or the monoamino compound **16** (0.20 mmol) plus 50 mg of fatty acid-free bovine serum albumin (Sigma) in 2.5 ml of water. The mixtures (unadjusted pH's 6.8 and 6.1, respectively) were gently stirred at room temperature for 6 h.

The clear solution (A) from the diamino reaction mixture was decanted from the oil (B) adhering to the side of the flask into a 30 × 1.2 cm tube of 0.002-in. cellulose membrane and was dialyzed against distilled water for 48 h. The water was changed every 12 h. Controlled trials showed that no diamino (or monoamino) material remained in the dialysis tube after 6–8 h. The above-mentioned viscous, water-insoluble oil (B) was dissolved in 1% aqueous sodium dodecylsulfonate (2 ml) and was dialyzed separately. After 48 h, this dialysis mixture contained some solid. The two dialyzed solutions were lyophilized separately to give 21 mg of dry coupled protein **11** from the soluble fraction A and 34 mg from the oily fraction B. The former fraction decreased markedly in solubility after lyophilization, since it now proved to be insoluble in water or in 1% aqueous sodium dodecylsulfonate, as well as in buffers from pH 2 to 8.1.

In the monoamino coupling, the originally clear reaction mixture eventually deposited a viscous material sticking to the glass. The aqueous portion was dialyzed as described above, and the oily portion as a solution in 1:1 dioxane–water was dialyzed separately. After dialysis, the contents of the two tubes, both of which now contained small amounts of precipitate, were lyophilized separately. The water-soluble fraction yielded 28 mg, the dioxane–water-soluble fraction 22 mg, of powdery coupled products. The solubility of the first fraction decreased sharply after lyophilization, since it now was practically insoluble in water, dilute detergent, or buffers at pH 4–8.1. The material was reasonably soluble, however, at pH 2.

Ultraviolet absorption measurements served to determine the number of prosthetic groups coupled to the protein. Ethyl *p*-acetamidobenzoate, mp 105–107 °C (lit.[17] mp 103–104 °C), was taken as an appropriate comparison standard. A useful absorption difference between bovine serum albumin and the *p*-acetamidobenzoate, both in 1:10 dioxane–water, was found at 260 nm, where the protein (mol wt 66 000) had ε 26 × $10^3$ and the benzoate had ε 7 × $10^3$. With this information, the apparent molecular extinction coefficients at 260 nm of the coupled proteins **11** and **17**, also in 1:10 dioxane–water, showed that the protein coupled to both ends of trehalose carried an average of 7 (fraction A) and 2.5 (fraction B) trehalose units per protein molecule, and that the protein coupled only to the 6 position carried an average of 4–5 trehalose units per protein molecule.

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**Registry No.**—**2**, 99-20-7; **3**, 42390-78-3; **4**, 59578-12-0; **5**, 60065-00-1; **6**, 60065-01-2; **7**, 60065-02-3; **8**, 60084-56-2; **9**, 60065-03-4; **10**, 60065-04-5; **12**, 60065-05-6; **13**, 60065-06-7; **14**, 60065-07-8; **15**, 60065-08-9; **16**, 60065-09-0; trimethylsilyl chloride, 75-77-4; *p*-nitrobenzoic acid, 62-23-7; *p*-nitrobenzoyl chloride, 122-04-3.

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(16) **Note Added in Proof.** After this paper was submitted for publication, we became aware of work by R. Toubiana, B. C. Das, J. Defaye, and B. Mompon, *Carbohydr. Res.*, **44**, 308 (1975), in which the same hexasilylated compound **4** was prepared by selective desilylation of octasilylated trehalose **3** with potassium carbonate in methanol. The reported melting was 115–118 °C in agreement with our value.
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# A Stereospcific Synthesis of Biotin via Thiophene Intermediates[1a]

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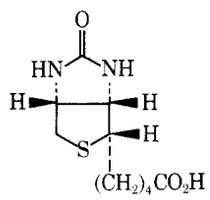
A total synthesis of the vitamin biotin (**1**) is described. Catalytic hydrogenation of the easily prepared thiophene **22** was found to occur stereospecifically and proceed in excellent yield. This approach features a selective ring closure of the amino diacid **6** to the eight-membered lactam **7**. A number of interesting rearrangements were discovered during the course of a modified Curtius reaction involving the mixed anhydride **16**, which led to the key aromatic substrate for reduction. A novel and efficient ring closure of the mixed diurethane **24** to the imidazolidone moiety of biotin was used to complete the synthesis.

Initially, the biological activity of biotin (**1**), a member of the B vitamin complex, was confined to its prevention of dermatitis and other degenerative effects in experimental animals.[1b] In recent years, however, researchers have discovered many new applications of this natural product in the areas of nutrition and growth promotion.[2] These findings have generated a renewed interest in the total synthesis of biotin, and this has led to the development of several new syntheses.[3,4]



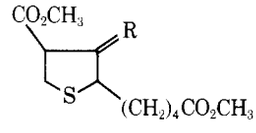

**1**

An examination of the structure of biotin (**1**) reveals the presence of three contiguous asymmetric centers, which requires a high degree of stereocontrol over synthetic intermediates. In addition, the three substituents on the tetrahydrothiophene ring are present in the thermodynamically least stable all-cis configuration.

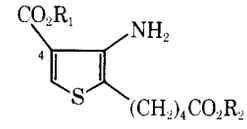
Recently, we have disclosed a solution to this problem which involved a novel oxidative rearrangement of an olefinic thiazolidine.[4] Earlier workers[5] have employed catalytic hydrogenation of a dihydrothiophene in this regard with varying degrees of success. Their efforts were often complicated by a lack of stereospecificity in the reduction step as well as other complications related to the chemistry of dihydrothiophenes. Therefore, it seemed reasonable that a synthesis of biotin based on readily available aromatic intermediates would offer several advantages. For example, the thiophene ring can be considered to be a protecting group for sulfur during the elaboration of the ring substituents. Furthermore, this protection may be dismantled by catalytic hydrogenation, conditions which in principle can simultaneously introduce the all-cis ring hydrogens of biotin in one operation. To date, no synthetically useful approach to biotin based on this concept has been reported,[6] reflecting the marked resistance of thiophenes to reduction.[7]

In this report we describe a highly stereospecific synthesis of biotin which incorporates an efficient reduction of the aromatic precursor **22** to the requisite oxidation level with concomitant introduction of the three cis hydrogens.

An easy entry into the appropriately substituted thiophenes begins with the readily available ketone **2**, prepared in large quantities from pimelic acid and methyl mercaptopropionate.[8] Treatment of **2** with hydroxylamine in pyridine at room temperature yielded the corresponding oxime **3** in quantitative yield. The two units of unsaturation present in the oxime functionality were induced to migrate into the thiophane ring system by simply dissolving the oxime **3** in ether saturated with hydrogen chloride for 24 h.[9] This rearrangement, which seems to require an electron-withdrawing group in position $\alpha$ to the oxime, afforded in 96% combined yield a mixture of the amino diester **4** and the corresponding amino acid **5**, in a ratio of 6:1. The water derived from the oxime dehydration presumably is the source of the by-product **5**, which is easily isolated by simple extraction.




**2**, R = O
**3**, R = NOH




**4**, $R_1 = R_2 = CH_3$
**5**, $R_1 = CH_3$; $R_2 = H$
**6**, $R_1 = R_2 = H$; $\cdot HCl$

Our synthetic plan at this point required that a Curtius reaction be carried out on the aromatic carbomethoxy group attached to C(4). Treatment of the amine diester **4** with hydrazine failed to distinguish between the two esters. Although the amino acid **5** carried the requisite differentiated groups,