Since all samples except αNO (pH 6.0) display the CC′ pair along g_3, it is tempting to extrapolate from the HbNO and βNO findings in D_2O that this group should be exchangeable in the other specimen too. Provided its correct assignment to the proton of the distal histidine N_{ϵ_2} nitrogen, some interesting implications pertain. First one notes that this coupling is strongest for the β chains (2.0 MHz) and smallest for αNO (pH 7.4) and MbNO (1.85 and 1.8 MHz, respectively), indicating a difference in coordination strength of the ligand by the distal histidine (E7). Moreover, this coordination is apparently lost in the α chains upon a tertiary transition to the t state, whereas the interaction remains in the β chains, providing for a substantial structural difference between the two. These findings may be connected to ESR work on NO-ligated mutant hemoglobins, which shows that the quaternary T structure is stabilized in the absence of the distal histidine in the α chains (Hb_{Boston} and $Hb_{Opossum}$) whereas the lack of His E7 in the β chains ($Hb_{Saskatoon}$) appears to have little influence on the quaternary state.[17,38,39]

It is difficult to arrive at a definite structural assignment for the other proton ENDOR lines, which all are from nonexchangeable hydrogens. Single-crystal studies that should clarify some of the assignments are being performed for MbNO presently. For example, the other two dipolar components of the pair CC′ on g_1 and g_2 could not be safely traced in HbNO and βNO by comparison between H_2O and D_2O at high pH, although some indications were found in the HbNO spectrum pointing toward a value of about 1 MHz or less. Also, the conspicuous appearance of pair BB′ (~1.4 MHz) for nearly all samples on all g turning points suggests the existence of a proton with a fairly large isotropic contribution that we cannot so far rationalize. Looking for feasible candidates for dipolar interactions of the observed size, we obtain a good fit for the pair DD′ along g_3 with one of the methyl protons C_{γ_1} of valine E11 (cf. Figure 7). Assuming that one of the protons points directly toward the nitrogen of the NO ligand, a distance of 2.1 Å can be calculated. Estimating the angle φ between the direction of g_3 and the vector (C_{γ_1})–H···N(NO) yields $\varphi = 75°$. With about 60% spin density, ρ, on N of NO[4,16] one then obtains from eq 4 $a_H = 3.8$ MHz, which is in surprising agreement with the typical values 3.4–3.85 MHz obtained. A survey of the nearest protons of both the proximal and the distal histidines in relation with either iron or the ligand atoms yields no satisfying alternative coincidence.

The assignment of the pair DD′ on g_3 to a valine proton, though tentative at this stage, derives support from the crystallographic data from which an interaction of valine E11 with the heme has been concluded.[36] The β chains parallel to the situation for the distal histidine show no significant difference in coupling strength of pair DD′ between the tertiary r and t states. The α chains appear to lose the interaction at low pH. Correcting, however, for the spin density change on the ligand NO between the two tertiary states it appears possible that the pair GG′ on g_3 (2.65 MHz) in αNO (pH 6.0) could be the corollary of the DD′ pair found for α chains in their tertiary r state.

If we adopt the position that the lines BB′ on g_1 and g_2 are the other dipolar elements of the DD′ pair on g_3, one still has to explain the occurrence of pairs of similar magnitude on g_2 and g_3. Taking the group DD′ on g_2 as a maximum dipole interaction, we find no agreement for it with any of the possible candidates in the heme pocket. The same holds the pair FF′ on g_2, which has a rather large (~5.2 MHz) coupling that we are unable to account for. It is interesting to note that this pair is found only in samples containing the α chains in the t state (αNO (pH 6.0) and HbNO + IHP) and in MbNO. The assignment of these groups has to await further studies.

Conclusions

ENDOR measurements on ^{14}N–His (F8) and ^1H couplings of NO-ligated hemoglobin and its derivatives give clear evidence for structural changes occurring in the heme environment of only the α chains on either a tertiary r–t transition of the isolated subunit or upon a quaternary change of the tetrameric Hb molecule. These involve the loss of the coupling of the proximal histidine (His F8) and most probably of the distal histidine (E7) N_{ϵ_2}. Although the large amount of proton lines observed in all samples needs further investigations on hybrids and Mb single crystals to fully elucidate the heme environment stereochemistry, the gamut of spectral variations obtained in this study allows the conclusion that the control of the structural state, both tertiary and quaternary, by the globin part is distinctly different in the heme environment of α and β chains.

Acknowledgment. M.H. is grateful to the DAAD for providing a short-term fellowship for a stay in Amherst. We are grateful to Prof. Dr. K. Gersonde (Aachen, FRG) for many stimulating discussions. The help of Prof. Dr. C. Bauer and Dr. H. Zorn providing us with IEF facilities and a sample of hemin DME as well as their frequent and helpful discussions are gratefully acknowledged. Thanks are also due to T. Provost, who has been very helpful in the initial preparation stages.

Registry No. $HisF_8$, 71-00-1; nitrosylhemin DME, 58357-23-6; nitrosylhemoglobin A, 52228-24-7; L-valine, 72-18-4.

(38) John, M. E.; Waterman, M. R. *J. Biol. Chem.* **1980**, *255*, 4501–4506.
(39) John, M. E.; Waterman, M. R. *J. Biol. Chem.* **1979**, *254*, 11953–11957.

Communications to the Editor

Preparation of a Mixture of Nucleoside Triphosphates from Yeast RNA: Use in Enzymatic Synthesis Requiring Nucleoside Triphosphate Regeneration and Conversion to Nucleoside Diphosphate Sugars[1]

Chi-Huey Wong, Sharon L. Haynie,[2] and George M. Whitesides*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

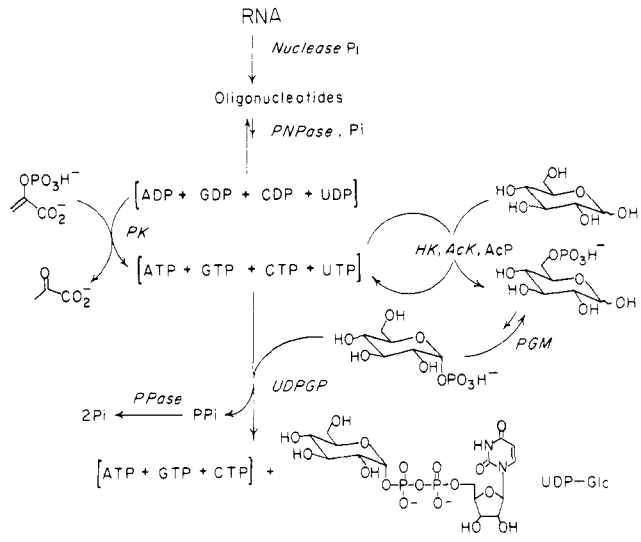
Received June 14, 1982

This paper describes a practical procedure for converting yeast RNA into a mixture of ATP, UTP, GTP, and CTP (Scheme I). This mixture can be used as a source of nucleoside triphosphates for the synthesis of nucleoside diphosphate sugars. These latter substances are required in most enzyme-catalyzed syntheses of oligo- and polysaccharides.[3] In addition, since many ATP-utilizing enzymes (especially phosphotransferases) will also accept GTP, UTP, and CTP,[4] the mixture of nucleoside triphosphates serves

* Inquiries should be addressed to G.M.W. at the Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, MA 02138.
(1) Supported by the National Institutes of Health, GM-26543 and GM-30367.
(2) National Science Foundation Predoctoral Fellow.
(3) Nikaido, H.; Hassid, W. Z. *Adv. Carbohydr. Chem. Biochem.* **1971**, *26*, 351–483. Rosevear, P. R.; Numez, H. A.; Barker, R. *Biochemistry* **1982**, *21*, 1421–31.

0002-7863/83/1505-0115$01.50/0

Scheme I[a]



[a] PNPase, polynucleotide phosphorylase; PK, pyruvate kinase; HK, hexokinase; AcK, acetate kinase; PGM, phosphoglucomutase; UDPGP, UDP-glucose pyrophosphorylase; PPase, inorganic pyrophosphatase; AcP, acetyl phosphate.

as a convenient source of phosphate equivalents in other enzyme-catalyzed organic syntheses.[5]

Conversion of RNA to the mixture of nucleoside triphosphates involved two steps. In the first, nuclease P_1 (E.C. 3.1.4.–, in solution) was used to hydrolyze high molecular weight yeast RNA to a mixture of lower molecular weight oligonucleotides.[6] In the second, polynucleotide phosphorylase (PNPase, E.C. 2.7.7.8, immobilized in PAN gel[7]) converted these oligonucleotides into nucleoside diphosphates by reaction with phosphate. This equilibrium conversion strongly favors oligonucleotides.[8] The nucleoside diphosphates were converted to nucleoside triphosphates by phosphorylation in situ by using phosphoenol pyruvate (PEP)[9] and pyruvate kinase (PK, E.C. 2.7.1.40) to drive the reaction.[10] The most expensive enzyme in this synthesis is PNPase ($4.00/unit, Sigma). The initial treatment by nuclease P_1 permits use of PNPase immobilized in PAN gel, in which form the enzyme is both relatively stable and very easily recovered for reuse.

In a representative procedure, yeast RNA (15 g, 95% pure, from Boehringer Mannheim) and 50 units of nuclease P_1 in 20 mL of water (0.1 mM in $MnCl_2$, pH 6.0) was stirred at 50 °C for 1 h. The mixture was adjusted immediately to pH 8.2 by adding cold 5 N NaOH solution and diluted to a total volume of 1 L in a solution that contained potassium phosphate (0.2 M), $MgCl_2$ (2mM), PEP (monopotassium salt, 80 mM), and PAN-immobilized PNPase (8 units in 20 mL of gel)[11] and PK (50 units in 1 mL of gel). The reaction mixture was stirred at room temperature under argon for 4 days, and the pH was controlled between 8.2 and 8.5. Enzymatic analysis[12] of the reaction mixture indicated that it contained 30 mmol of nucleoside triphosphates (68% yield based on RNA); no further increase in the yield of these substances was observed[13] with longer reaction times. The gel particles were removed, the solution was adjusted to pH 3.0 at 4 °C by adding cold concentrated HCl with stirring, and a precipitate was removed by filtration. The filtrate was adjusted to pH 8.0 (5 N NaOH) and concentrated under reduced pressure at 35 °C to a volume of 50 mL. This solution contained a mixture of nucleoside triphosphates (28 mmol, 62% yield based on RNA) in these relative quantities:[14] ATP, 24%; UTP, 28%; GTP, 30%; CTP, 18%. The enzymatic activities recovered after these transformations were PNPase, 75%, and PK, 92%.

The mixture of nucleoside triphosphates obtained in this procedure is contaminated with a number of other components. These other materials do not, however, seem to interfere with the use of the nucleoside triphosphates in further enzymatic processes. In particular, the conversion of the UTP present in the mixture to UDP-glucose and the use of the components of the mixture to act as cofactors in the hexokinase-catalyzed phosphorylation of glucose[15] were both uneventful.

For the synthesis of UDP-glucose (UDP-Glc), 40 mL of the mixture of cofactor (6.2 mmol of UTP) was diluted to 200 mL (pH 8.0). PAN-immobilized UDP-Glc pyrophosphorylase (UDPGP, E.C. 2.7.7.9, 50 units in 1 mL of gel), inorganic pyrophosphatase (PPase, E.C. 3.6.1.1, 60 units in 0.5 mL of gel), phosphoglucomutase (PGM, E.C. 2.7.5.1, 52 units in 1 mL of gel), and glucose 6-phosphate (G-6-P, generated from 6.2 mmol of barium salt by treating with Dowex 50 to remove barium ion)[15] were added. The reaction was conducted under argon for 20 h with pH controlled at 7.5. Enzymatic analysis[16] of the solution indicated it contained 6 mmol of UDP-Glc and 14 mmol of a mixture of other nucleoside triphosphates. After separation of the enzyme-containing gel, the solution could be used directly for UDP-Glc—requiring disaccharide synthesis,[17] or isolated by chromatography using Bio-Rad P-2 (H_2O solvent).[18]

The procedure summarized in Scheme I provides the best method presently available for the preparation of GTP, UTP, and CTP, provided that the *mixture* of nucleoside triphosphates is acceptable. We have explored an alternative scheme for generation of this mixture based on nuclease P_1-catalyzed hydrolysis of RNA to a mixture of nucleoside *mono*phosphates, followed by phosphorylation of these species to triphosphates.[19] We have not found the nucleoside monophosphate kinases required for this scheme to be either easily prepared or easily handled.

The major weakness of the scheme described here is the present expense of commercial PNPase. We note, however, that this enzyme is readily available from *E. Coli* B[20] and could in any event

(4) Bergmeyer, H. U. In "Methods of Enzymatic Analysis"; Academic Press: New York, 1974; p 2081. Chung, A. E. *J. Biol. Chem.* **1967**, *242*, 182–6. Plowman, K. M.; Krall, A. R. *Biochemistry* **1965**, *4*, 2809–25. These enzymes include hexokinase, phosphoglycerate kinase, acetate kinase, pyruvate kinase, phosphofructokinase, NAD kinase, glycerokinase, and glucose 1-phosphate kinase.

(5) Pollak, A.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 2366–7. Rios-Mercadillo, V. M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1979**, *101*, 5828–9.

(6) Commercial yeast RNA contains a high proportion of polynucleotides with M_n 10^5–10^6. This material was not a substrate for PAN-immobilized PNPase and was a poor substrate for this enzyme in soluble form. After digestion with nuclease P_1, it became a good substrate for immobilized PNPase.

(7) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–36.

(8) Godefroy-Colburn, T.; Grumberg-Manago, M. In "The Enzymes"; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 7, pp 533–74. At 20 °C, 80% of ADP present initially is polymerized, corresponding to an equilibrium constant $K = 16$ for the reaction ADP + ApA $\rightleftharpoons$ ApApA + Pi ($\Delta G^{\circ\prime} = -RT \ln K \simeq -1.7$ kcal/mol).

(9) Hirschbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765–6.

(10) If the reaction ApApA + Pi $\rightleftharpoons$ ApA + ADP ($\Delta G^{\circ\prime} = +1.7$ kcal/mol) is coupled with the reaction PEP + ADP $\rightleftharpoons$ ATP + pyruvate ($\Delta G^{\circ\prime} = -7.5$ kcal/mol), the free energy ($\Delta G^{\circ\prime}$) for the overall reaction ApApA + Pi + PEP $\rightleftharpoons$ ApA + ATP is −5.8 kcal/mol. Saber, H. A. "Handbook of Biochemistry"; **1970**, The Chemical Rubber Co.: Cleveland, OH, 1970; pp J180–5.

(11) The enzyme PNPase from *M. Lysodeikticus* was immobilized according to the standard procedure.[7]

(12) Determined by PEP and PK coupled with lactic dehydrogenase.

(13) Mononucleotides, dinucleotides, polynucleotides with therminal 3′-phosphate groups, and double-stranded polynucleotides are not substrates of PNPase: Godefroy, T. *Eur. J. Biochem.* **1979**, *14*, 222–31. Godefroy, T.; Cohn, M.; Grunberg-Manago, M. *Ibid.* **1970**, *12*, 236–49. Chou, J. Y.; Singer, M. F. *J. Biol. Chem.* **1970**, *245*, 995–1004.

(14) HPLC was performed by using a Waters Radial-PAK C_{18} column (5-mm i.d.) with 5 mM tetrabutylammonium phosphate in aqueous acetonitrile (18% v/v, H 7.6) as the mobile phase.

(15) Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890.

(16) Determined enzymatically with UDP-glucose dehydrogenase and NAD; see Bergmeyer.[4]

(17) Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 5416–5418.

(18) Previous preparations of UDP-glucose: Moffat, J. G. *Methods Enzymol.* **1968**, *8*, 136–42. Kawaguchi, K.; Kawai, H.; Tochikura, T. *Methods Carbohydr. Chem.* **1980**, *8*, 261–9.

(19) Leuchs, H. J.; Lewis, J. M.; Rios-Mercadillo, V. M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1979**, *101*, 5829–30.

probably be made in quantity by recombinant DNA methods.

Registry No. ATP, 56-65-5; UTP, 63-39-8; GTP, 86-01-1; CTP, 65-47-4; nuclease P_1, 54576-84-0; PNPase, 9014-12-4; PEP, 138-08-9; PK, 9001-59-6; UDP-Glc, 133-89-1; glucose, 50-99-7; G-6-P, 56-73-5.

Supplementary Material Available: Procedures for preparation of glucose-6-phosphate by using the XTP's prepared here and for immobilization of PNPase (1 page). Ordering information is given on any current masthead page.

(20) Kimhi, Y.; Littauer, U. Z. *Methods Enzymol.* **1968**, *12B*, 513–9. Starting from 540 g of the frozen cells, 880 units of PNPase were detected. After several steps of purification, 150 units of the enzyme were isolated with specific activity 6 unit/mg (1 unit will generate 1 μmol of ATP/min from poly(A) in a coupled reaction).

Asymmetric Alkylation of α-Amino Carbanions. An Enantioselective Synthesis of (*S*)-1-Alkyl-1,2,3,4-tetrahydroisoquinolines

A. I. Meyers* and Lelia M. Fuentes

Department of Chemistry, Colorado State University
Fort Collins, Colorado 80523

Received October 18, 1982

Asymmetric C–C bond-forming reactions have reached a high level of efficiency over the past 8 years, thus adding asymmetric synthesis to the arsenal of tools available to the organic chemist for obtaining chiral compounds.[1] Absent from these recent successes is the introduction of a C–C bond adjacent to nitrogen with simultaneous enantioselectivity, a transformation that would provide access to many alkaloids in enantiomeric form. On the basis of our earlier reports[2] describing α-amino carbanions derived from the formamidines (A–C, among others), we sought to introduce in place of the *N*-*tert*-butyl group an appropriate chiral auxiliary that would effect the diastereofacial selectivity during the alkylation of the lithio carbanion. We can now report the results of a highly successful asymmetric alkylation leading to 1-substituted tetrahydroisoquinolines in >90% enantiomeric excess with predictable absolute configuration (Table I). The enantiomeric alkylation was initially investigated by using (*R*)-(−)-α-phenethylamine (PEA) as the chiral auxiliary. Thus, tetrahydroisoquinoline **1** (Scheme I) was transformed into its *N*-formyl derivative **2**[3] and treated with Meerwein's reagent and (−)-PEA, which gave the formamidine **3a** [98%, $[\alpha]_D$ −64.94° (*c* 3.4, THF)]. Metalation of **3** with lithium diisopropylamide (1.2 equiv, THF, −78 °C) followed by addition of various alkyl halides gave the alkylated product **4a**, which was directly subjected to hydrazinolysis affording **5**. As seen from Table I, although the chemical yields (**3a** → **5**) were generally quite high, the enantioselectivity of the process was only moderate (10–52% ee). Furthermore, the absolute configurations for **5** were not consistent, the first four entries in the table giving *S* as the predominant enantiomer while the last appearing as the *R* enantiomer.[4]

We next turned to the chiral auxiliary derived from (1*S*,2*S*)-(+)-1-phenyl-2-amino-1,3-propanediol previously em-

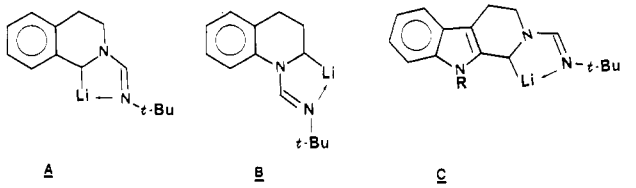
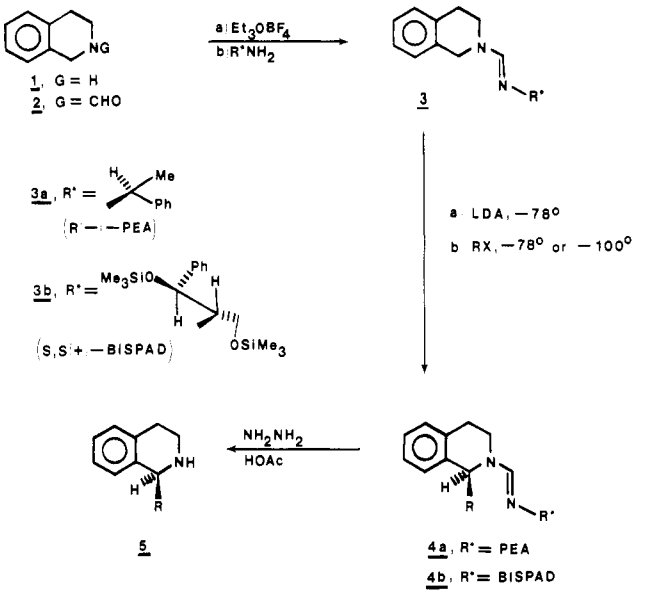


Table I. Asymmetric Alkylation of Chiral 1,2,3,4-Tetrahydroisoquinolines, 3

RX[a]	1-alkyltetrahydroisoquinolines 5: chem yield, %[c]	$[\alpha]_D^{24}$ (EtOH),[d] deg	ee,[e] %	confn[e]
3a (R = (*R*)-(−)-PEA)				
MeI	85	+0.90	10	*R*
i-BuBr	84	+13.67	27	*R*
n-BuBr	93		19	*R*
$PhCH_2Br$	97	+13.00	35	*R*
$PhCH_2CH_2Br$	89		52	*S*
3b (R = (*S,S*)-(+)-BISPAD)				
MeI	80	−7.25	80	*S*
MeI[b]	79	−8.96	>99	*S*
i-BuI[b]	85	−47.21	91	*S*
n-BuBr[b]	80	−50.30	91	*S*
$PhCH_2Br$[b]	70	−35.71	93	*S*
$PhCH_2CH_2Br$[b]	65	−38.69	>99	*S*

[a] Alkyl halides added at −78 °C unless otherwise noted. [b] Alkyl halides added at −100 °C (liquid N_2–MeOH). [c] Based on formamidines **3a** or **3b**. [d] Rotations are for hydrochloride salts, since the free bases tend to air-oxidize slowly on standing. [e] Determined by preparing the 1-naphthoyl amides of **5** (1.5 equiv of 1-naphthoyl chloride, dichloromethane) and by purification by radial chromatography. This was injected onto the covalent phenylglycine-modified Spherisorb S5NH column (Regis Chemical Co.) incorporated in a Waters 440 HPLC instrument. For details of this technique see: Pirkle, W. H.; House, D. W.; Finn, J. M. *J. Chromatog.* 1980, *192*, 143. The elution solvent was 10% isopropanol–hexane, and the integration of the peaks (254 nm) was based on ratios of previously injected racemates of naphthoyl amides of **5**. The absolute configurations are assigned by order of elution and comparison with (*S*)(−)-7.

Scheme I



(1) Asymmetric C–C bond-forming reactions have been summarized in part: Eliel, E.; Otsuka, S. In "Asymmetric Reactions and Processes in Chemistry"; American Chemical Society: Washington, D.C., 1982; ACS Symp. Ser., No. 185. For additional recent achievements in asymmetric C–C bond forming reactions see: (a) Evans, D. A.; Bartoli, J. *Tetrahedron Lett.* **1982**, *23*, 807. (b) Schollkopf, U.; Groth, U. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 977. (c) Enders, D.; Eichenauer, H. *Chem. Ber.* **1980**, *112*, 2933. (d) Houge, C.; Frisque-Hesbain, A. M.; Mockel, A.; Ghosez, L.; DeClercq, J. P.; Germain, G.; Van Meerssche, M. *J. Am. Chem. Soc.* **1982**, *104*, 2921. (e) Meyers, A. I.; Williams, D. R.; Erickson, G. W.; White, S.; Druelinger, M. *Ibid.* **1981**, *103*, 3081, 3088.

(2) (a) Meyers, A. I.; ten Hoeve, W. *J. Am. Chem. Soc.* **1980**, *102*, 7125. (b) Meyers, A. I.; Hellring, S.; ten Hoeve, W. *Tetrahedron Lett.* **1981**, *22*, 5115. (c) Meyers, A. I.; Hellring, S. *Ibid.* **1981**, *22*, 5119. (d) Meyers, A. I.; Hellring, S. *J. Org. Chem.* **1982**, *47*, 2229.

(3) Ugi, I.; Meyr, R.; Lipinski, M.; Bodesheim, F.; Rosendahl, F. *Org. Synth.* **1961**, *41*, 13.

(4) A large number of racemic α-naphthoyl amides of secondary amines have been separated by using the chiral stationary phase column developed by Pirkle (footnote e, Table I), and absolute configurations have been verified by order of elution. This is the subject of a separate report to be published.

0002-7863/83/1505-0117$01.50/0