

Cell Surface Engineering by a Modified Staudinger Reaction

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Selective chemical reactions enacted within a cellular environment can be powerful tools for elucidating biological processes or engineering novel interactions. A chemical transformation that permits the selective formation of covalent adducts among richly functionalized biopolymers within a cellular context is presented. A ligation modeled after the Staudinger reaction forms an amide bond by coupling of an azide and a specifically engineered triarylphosphine. Both reactive partners are abiotic and chemically orthogonal to native cellular components. Azides installed within cell surface glycoconjugates by metabolism of a synthetic azidosugar were reacted with a biotinylated triarylphosphine to produce stable cell-surface adducts. The tremendous selectivity of the transformation should permit its execution within a cell's interior, offering new possibilities for probing intracellular interactions.

Chemoselective ligation reactions designed to modify only one cellular component among all others have provided insight into cellular processes (1). The goal in developing such transformations is to equal the tremendous selectivity of noncovalent recognition events, such as antibody-antigen binding, that direct many normal biological processes and are now powerful experimental tools. In order to achieve this, the two participating functional groups must have finely tuned reactivity so that interference with coexisting functionality is avoided. Ideally, the reactive partners would be abiotic, form a stable adduct under physiological conditions, and recognize only each other while ignoring their cellular surroundings. The demands on selectivity imposed by cells preclude the use of most conventional covalent reactions, and thus far only two have proven utility in a biological environment.

One chemoselective ligation reaction, that between a ketone and an aminoxy or hydrazide group, has enabled us to engineer the composition of cell surfaces (2). We introduced ketones onto cells through unnatural sialic acid biosynthesis. Human cells metabolize the unnatural precursor *N*-levulinoylmannosamine (compound 2, Fig. 1), a ketone-bearing analog of the native sugar *N*-acetylmannosamine (compound 1, Fig. 1), to the corresponding keto-sialic acid residues on cell surface glycoconjugates. Chemically orthogonal to native cell surface components, the ketone can then react selectively with externally delivered aminoxy or hydrazide reagents to form stable covalent adducts. Applications of this reaction include the chemical construction of new glycosylation patterns on cells (3), new approaches

to tumor cell targeting (4), and novel receptors for facilitating viral-mediated gene transfer (5).

Although useful for cell surface chemistry, ketone ligation reactions have limited intracellular use owing to competition with endogenous keto-metabolites. Tsien and co-workers reported a second chemoselective ligation reaction that circumvents this problem—condensation of a cysteine-rich hexapeptide motif with a bis-dithioarsolane (6). This enabled the targeting of a synthetic fluorescent dye to a single protein within the environs of a living cell. In order to augment existing chemical approaches to the study and manipulation of cellular components, the identification of new cell-compatible chemoselective ligation reactions is of fundamental importance. We have therefore focused on refining traditional chemical transformations in accordance with cellular demands.

The Staudinger reaction occurs between a phosphine and an azide to produce an aza-ylide (Fig. 2A) (7, 8). In the presence of water, this intermediate hydrolyzes spontaneously to yield a primary amine and the corresponding phosphine oxide. The phosphine and the azide react with each other rapidly in water at room temperature in high yield. Both are abiotic and essentially unreactive toward biomolecules inside or on the surfaces of cells. Thus, in its classical form, the Staudinger reaction meets many of the criteria required of a chemoselective ligation in a cellular environment. Where it falls short is that the initial covalent adduct, the aza-ylide, is not stable in water. Our solution to this problem was to design a phosphine that would enable rearrangement of the unstable aza-ylide to a stable covalent adduct. We envisioned that an appropriately situated electrophilic trap, such as a methyl ester, within the phosphine structure would capture the nucleophilic aza-ylide by intramolecular cyclization (Fig. 2B). This process, in turn, would ultimately produce a stable amide bond rather than the products of aza-ylide hydrolysis. We tested this

hypothesis in a model reaction (Fig. 2C) between a simple phosphine and methyl 2-azidogalactose in aqueous tetrahydrofuran (THF); only the ligation product was observed with no evidence of aza-ylide hydrolysis.

The cell surface is a far more demanding environment, and to test the modified Staudinger reaction in this context we required (i) a method of installing azides on cells and (ii) a water-soluble phosphine reagent. The azide was selected rather than the phosphine for cell surface display because of its small size and the synthetic accessibility of azidosugars as metabolic precursors. On the basis of our earlier work with *N*-levulinoylmannosamine, we predicted that *N*-azidoacetylmannosamine (compound 3, Fig. 1) (9) would be well tolerated by the sialic acid biosynthetic machinery. Biotinylated phosphine 5 (10) (Fig. 3A) was designed for water solubility, by virtue of the tetraethyleneglycol linker, and for detection of the ligated cell-surface product. The synthesis of compound 5 was performed as shown in Fig. 3A; a versatile intermediate, compound 4, bears a carboxylic acid to which any biological probe or biopolymer can be appended. The proposed reaction of 5 with cell surface azido-sialic acid is depicted in Fig. 3B.

Jurkat cells were incubated with *N*-azidoacetylmannosamine, in acetylated form (11), at a concentration of 20 μ M for 3 days. The cells were washed and then reacted with compound 5 [1 mM in phosphate-buffered saline (PBS), pH 7.4] for 1 hour. After staining with fluorescein isothiocyanate (FITC)-avidin, the cells were analyzed by flow cytometry (Fig. 4A). Jurkat cells treated with acetylated 3 showed a marked increase in fluorescence that indicated the accumulation of biotin moieties on the cell surface, whereas untreated cells showed only a background level of fluorescence after exposure to phosphine 5. The fluorescence signal was reduced by the addition of tunicamycin during incubation of Jurkat cells with the azidosugar, in agreement with previous observations that most sialic acids on Jurkat cells reside within *N*-linked glycans (3). The background fluorescence was identical to that observed with Jurkat cells that were not exposed to any reagents (12) and thus represents autofluorescence of cells and not nonspecific uptake of the biotin probe or FITC-avidin.

HeLa cells responded similarly to incubation with acetylated 3 followed by reaction with compound 5. Notably, HeLa cells that were cultured for an additional 3 days after the modified Staudinger reaction showed no change in growth rate. Thus, neither metabolism of azidosugars, reaction with phosphine 5, nor the covalent attachment of phosphine oxide adducts to the cell surface appears to affect cell viability.

Using biotinylated beads of known biotin

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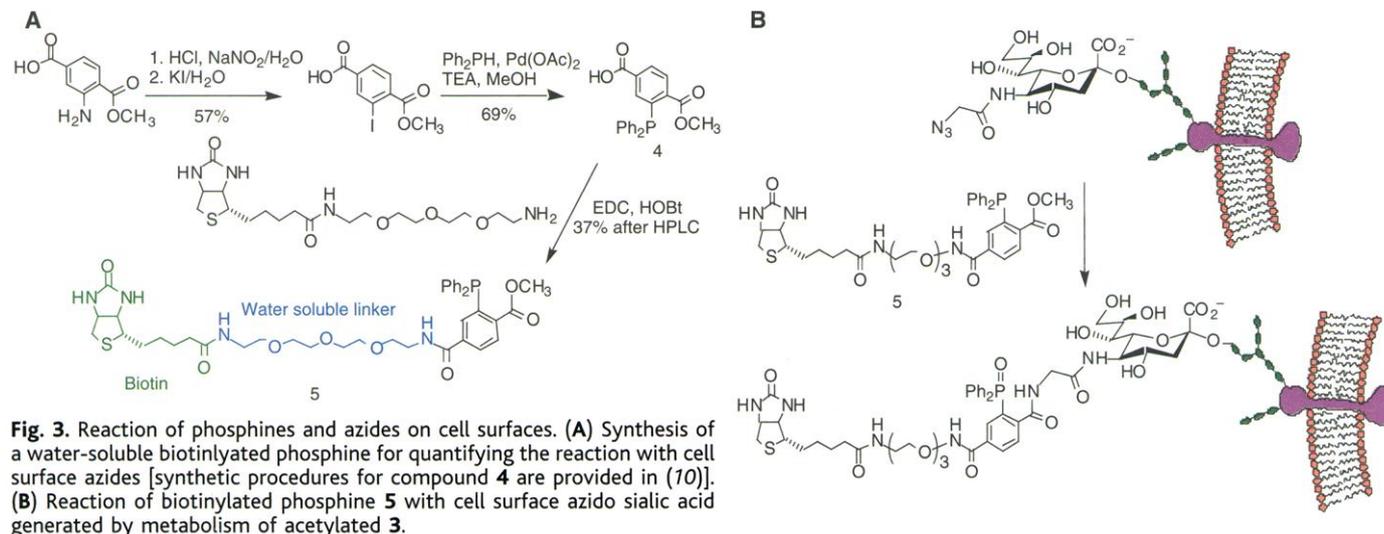
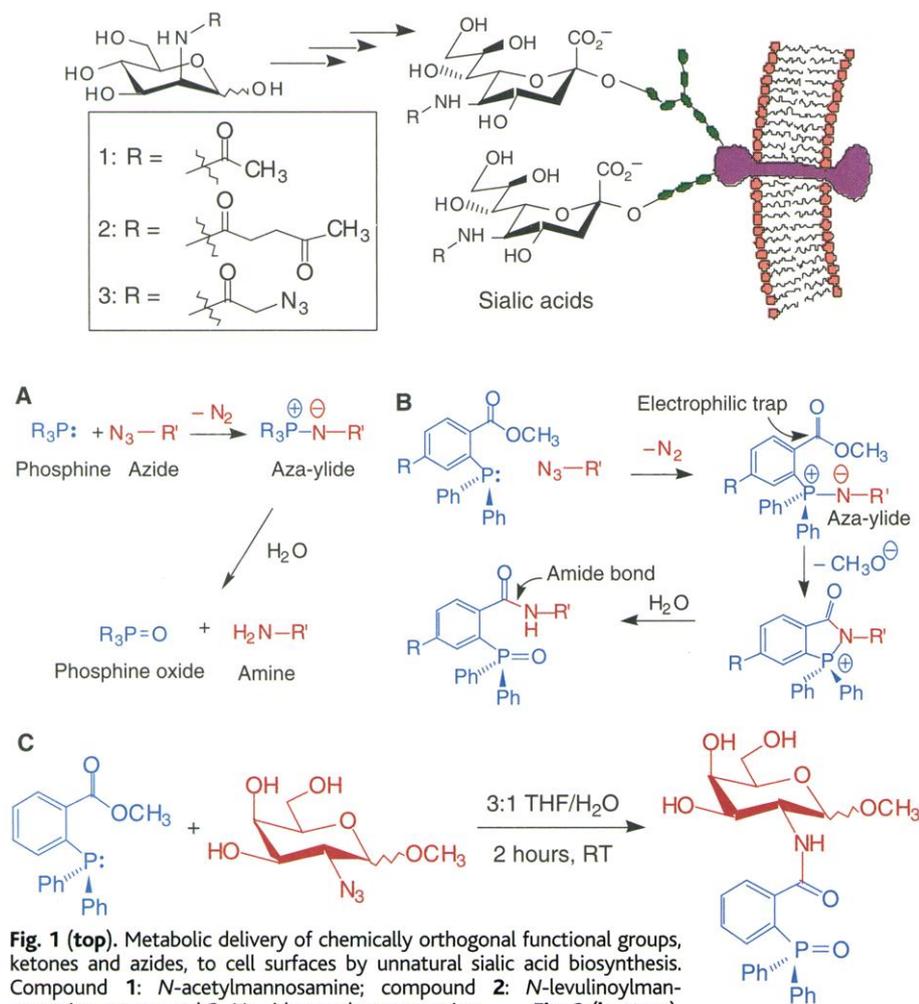
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density, we were able to correlate the fluorescence intensities observed by flow cytometry with the number of dye molecules on a particle

or cell (2). On this basis, we determined that Jurkat cells treated with 40 μM acetylated **3** for 3 days, followed by reaction with 1 mM com-

pound **5** for 1 hour, accumulated $\sim 850,000$ biotin moieties on the cell surface. This value places a lower limit on the number of azides present on the cell surface, as some azides may be concealed within the glycocalyx and therefore not accessible to the phosphine reagent. Furthermore, the cell surface reaction may not proceed in quantitative yield as observed with the model reaction (Fig. 2C). Higher densities of cell surface biotin moieties could be achieved by extending the reaction time as shown in Fig. 4B. Increasing the concentrations of the azidosugar or phosphine probe also elevated the level of cell surface modification. For example, Jurkat cells treated with 40 μM acetylated **3** for 3 days, followed by reaction with 2 mM compound **5** for 3 hours, accumulated ~ 4.5 million biotin moieties on the cell surface. We observed a dependence of the cell surface reaction yield on pH; reaction at pH 6.5 produced 75% of the fluorescence signal observed at pH 7.4 (Fig. 4C). This is consistent with previous observations that protonation of aza-ylides facilitates their hydrolysis, a competing side reaction of the modified Staudinger process (8).

We considered one alternative explanation for the azide-dependent localization of biotin on cells. Phosphine **5** might have reduced cell surface azides to the corresponding amines by the classical Staudinger reaction, simultaneously producing phosphine oxide **6** (Fig. 5A). Compound **6**, in turn, might nonspecifically acylate cell surface amines. If so, the reaction would lose the critical element of selectivity that we sought for biological applications. To address this possibility, we independently synthesized compound **6** and investigated its reactivity with cells. Two populations of Jurkat cells were pretreated with the azidosugar to engender cell surface azides. One population was then further reacted with a water-soluble trisulfonated triphenylphosphine to intentionally reduce the azides. In both cases, no cell surface biotinylation was observed. This result contrasts mark-



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edly with the extensive biotinylation of azido-sugar-treated cells reacted with phosphine **5** (Fig. 5A). We conclude that the chemoselective ligation reaction proceeds as designed without complications arising from nonspecific amine acylation.

To satisfy the requirement of chemical orthogonality, both participants in the reaction may not engage functional groups endogenous to cells. Triarylphosphines are mild reducing agents, which raises the possibility of disulfide bond reduction as an undesirable side reaction. We addressed this issue by incubating Jurkat cells with triarylphosphine **4**, an intermediate in the synthesis of **5**, and quantifying the appearance of free sulfhydryl groups on the cell surface with iodoacetylbiotin and FITC-avidin (Fig. 5B). After 1 hour in the presence of 1 mM **4**, no detectable increase in free sulfhydryl groups was observed relative to cells exposed to iodoacetylbiotin alone. In a positive control experiment, we incubated Jurkat cells with the trialkylphosphine TCEP (1 mM, 1 hour), a commercial disulfide bond reducing agent. A marked increase in free cell-surface sulfhydryl groups was observed in this case (Fig. 5B). We conclude that triarylphosphines such as **4** and **5** are essentially unreactive toward disulfide bonds under these conditions, rendering ligation with azides the predominant pathway for reactivity.

In a side-by-side comparison with our previously reported cell surface ketone reaction (2), the cell surface Staudinger process was superior in several respects. Using the same reagent concentrations, azidosugar metabolism followed by phosphine reaction produced two-fold higher fluorescence than ketosugar metabolism followed by hydrazide reaction. This may reflect either a faster reaction at the cell surface or more efficient metabolism of azidosugar **3** as compared with ketosugar **2** (Fig. 1). The azide has a major advantage over the ketone in that its reactivity is unique in a cellular context owing to its abiotic nature. Ketones, by contrast,

abound inside cells in the form of metabolites such as pyruvic acid and oxaloacetate. The modified Staudinger reaction is chemically orthogonal to ketone ligations and should allow tandem modification of cell surfaces with the two chemistries.

The susceptibility of azides to reduction during the metabolic process warrants some consideration in light of the reducing potential of the cell's interior. Monothiols such as glutathione can reduce alkyl azides at alkaline pH, but the rates of such reactions under physiological conditions are insignificant on the time scale of our experiments (13). Correspondingly, metabolic studies of the azido drug AZT (azidothymidine) showed 90% recovery of the azide, either in its administered form or metab-

olized to the glucuronidated compound, without significant reduction (14).

The delivery of azides to cell surfaces through other carbohydrate biosynthetic pathways could significantly expand applications of cell surface engineering. Azides and phosphines are abiotic structures both inside and outside cells, which raises the exciting possibility that their ligation could proceed in the intracellular environment. Given existing powerful methods for incorporating unnatural building blocks into other biopolymers, one need not be restricted to cell surface oligosaccharides as hosts for these chemical handles (15, 16). Azido-amino acids, for example, could be introduced into proteins and later targeted with phosphine probes. The

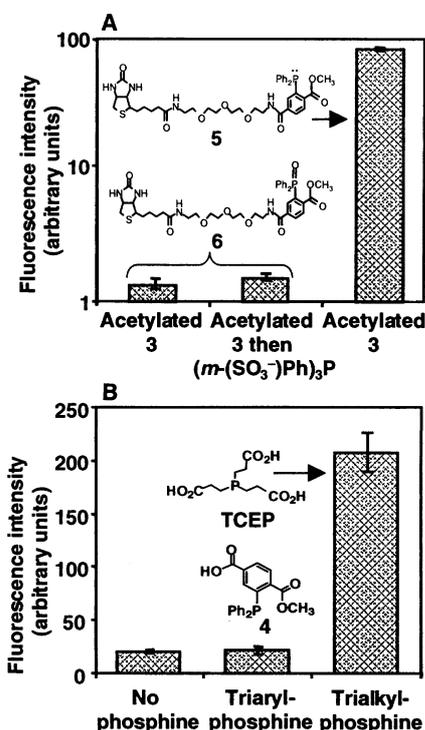
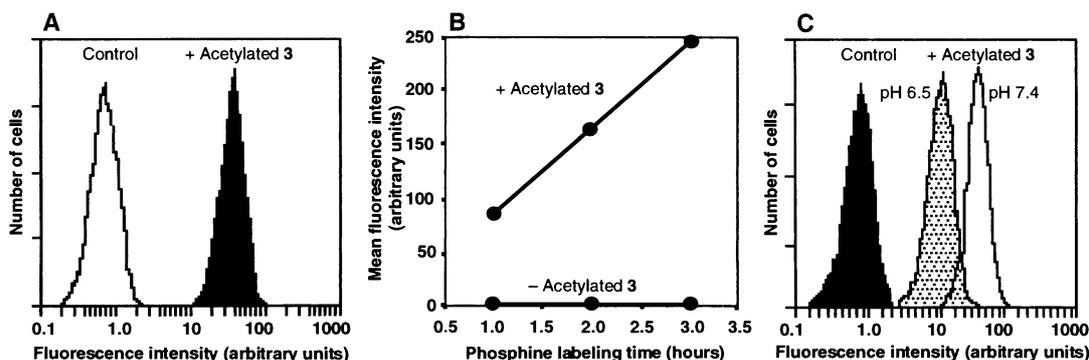


Fig. 5. Specificity of the modified Staudinger reaction. **(A)** Cell surface biotinylation does not proceed by classical Staudinger azide reduction followed by nonspecific acylation. Jurkat cells were cultured in the presence of acetylated **3** as described in Fig. 4. Cell surface azides were either reduced intentionally with a trisulfonated triphenylphosphine or left unreduced. Phosphine oxide **6**, the product of the classical Staudinger reaction, was prepared independently and incubated with the cells (1 mM for 1 hour). Analysis by flow cytometry was performed as in Fig. 4. **(B)** Triarylphosphines do not reduce disulfide bonds at the cell surface. Jurkat cells were incubated with a 1 mM solution of triarylphosphine **4** or TCEP for 1 hour at room temperature. The cells were centrifuged (2 min, 3000 g), washed with PBS, and diluted to a volume of 240 μ l. Samples were combined with 60 μ l of a solution of iodoacetylbiotin (5 mM in PBS). After incubation in the dark at room temperature for 1.5 hours, the cells were washed with buffer, stained with FITC-avidin, and analyzed by flow cytometry. In both (A) and (B), error bars represent the standard deviation of two replicate experiments.

Fig. 4. (A) Analysis of cell surface reaction by flow cytometry. Jurkat cells (1.25×10^5 cells per milliliter) were cultured in the presence or absence (control) of acetylated **3** (20 μ M for 3 days). The cells were washed twice with 1 ml of buffer (0.1% fetal bovine serum in PBS, pH 7.4) and diluted to a volume of 240 μ l. Samples were added to 60 μ l of a solution of **5** (5 mM in PBS, pH 7.4) and incubated at room temperature for 1 hour. The cells were washed and resuspended in 100 μ l of buffer, then added to 100 μ l of FITC-avidin staining solution (1:250 dilution in PBS). After a 10-min incubation in the dark at 4°C, the cells were washed with 1 ml of buffer and the FITC-avidin staining was repeated. The cells were washed twice with buffer, then diluted to a volume of 300 μ l for flow cytometry analysis. Similar results were



obtained in two replicate experiments. **(B)** Progress of reaction over time. Assays were performed as in (A) with 40 μ M acetylated **3** and varying the duration of the reaction with compound **5**. **(C)** pH profile of reaction. Assays were performed as in (A) with 40 μ M acetylated **3** and varying the pH of the buffer used during incubation with compound **5**.

introduction of the reactive partners into transiently associated biopolymers might allow their covalent trapping within a cell and, as a result, the identification of previously unobservable interactions.

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9. Synthesis of *N*-azidoacetylmannosamine (**3**) and acetylated **3**. A solution of mannosamine hydrochloride (250 mg, 1.16 mmol) and sodium methoxide (1.16 ml of a 1 M methanolic solution) in dry MeOH (10 ml) was stirred for 1 hour at room temperature, after which chloroacetic anhydride (991 mg, 5.80 mmol) was added. The resulting solution was stirred overnight at room temperature under an atmosphere of N₂ and then quenched with H₂O (5 ml) for 1 hour. The solution was neutralized with saturated NaHCO₃ and concentrated, and the residue was filtered through a plug of silica gel eluting with 5:1 CHCl₃/MeOH. The crude product obtained was dissolved in dimethylformamide (10 ml) and NaN₃ (78 mg, 1.39 mmol) was added. After heating at reflux overnight, the solution was cooled and concentrated. Purification by silica gel chromatography eluting with a gradient of 50:1 to 6:1 CHCl₃/MeOH afforded 179 mg of compound **3** (59% over two steps). The compound was peracetylated before incubation with cells as follows. A solution of **3** (25 mg, 0.095 mmol), acetic anhydride (1.0 ml, 11 mmol), and a catalytic amount of 4-dimethylaminopyridine in pyridine (2 ml) was cooled to 0°C. The mixture was stirred overnight, warmed to room temperature, then diluted with CH₂Cl₂ (100 ml) and washed with 1 N HCl (3 × 50 ml), saturated NaHCO₃ (1 × 50 ml), water (1 × 50 ml), and saturated NaCl (1 × 50 ml). The combined organic layers were dried over Na₂SO₄ and concentrated. The crude product was purified by silica gel chromatography eluting with a gradient of 1:10 to 1:2 EtOAc/hexanes to afford 39 mg (95% of acetylated **3**).
10. Synthesis of intermediate phosphine **4**. A solution of NaNO₂ (180 mg, 2.64 mmol) in 1 ml of H₂O was added dropwise to a solution of 1-methyl-2-amino-terephthalate (500 mg, 2.56 mmol) in 5 ml of cold concentrated HCl. The mixture was stirred for 30 min at room temperature and then filtered through glass wool into a solution of KI (4.30 g, 25.0 mmol) in 7 ml of H₂O. The dark red solution was stirred for 1 hour and then diluted with CH₂Cl₂ (100 ml) and washed with saturated Na₂SO₃ (2 × 10 ml). The organic layer was washed with water (2 × 20 ml) and saturated NaCl (1 × 20 ml). The combined aqueous layers were back extracted with CH₂Cl₂ (20 ml). The combined organic layers were dried over Na₂SO₄ and concentrated. The crude product was dissolved in a minimum amount of MeOH and H₂O was added until the solution appeared slightly cloudy. Cooling to 4°C and subsequent filtration afforded 449 mg (57%) of a yellow solid. To a flame-dried flask was added this product (300 mg, 1.00 mmol), dry MeOH (3 ml), triethylamine (0.3 ml, 2 mmol), and palladium acetate (2.2 mg, 0.010 mmol). While stirring under an atmosphere of Ar, diphenylphosphine (0.17 ml, 1.0 mmol) was added to the flask by means of a syringe. The resulting solution was heated at reflux overnight, and then allowed to cool to room temperature and concentrated. The residue was dissolved in 250 ml of a 1:1 mixture of CH₂Cl₂/H₂O and the layers were

separated. The organic layer was washed with 1 M HCl (1 × 10 ml) and concentrated. The crude product was dissolved in a minimum amount of methanol and an equal amount of H₂O was added. The solution was cooled to 4°C for 2 hours and the resulting solid was collected by filtration. The pure product, compound **4**, was isolated in 69% yield (245 mg). This compound can be coupled with amines by using standard procedures [such as EDC [1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride] or DCC (1,3-dicyclohexylcarbodiimide) coupling reactions].

11. Acetylated monosaccharides are metabolized 200-fold more efficiently than the free sugars owing to improved cellular uptake, which is followed by deacetylation by cytosolic esterases [C. L. Jacobs and C. R. Bertozzi, unpublished results; A. K. Sarkar, T. A. Fritz, W. H. Taylor, J. D. Esko, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3323 (1995)].

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1 December 1999; accepted 2 February 2000

A Fossil Snake with Limbs

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A 95-million-year-old fossil snake from the Middle East documents the most extreme hindlimb development of any known member of that group, as it preserves the tibia, fibula, tarsals, metatarsals, and phalanges. It is more complete than *Pachyrhachis*, a second fossil snake with hindlimbs that was recently portrayed to be basal to all other snakes. Phylogenetic analysis of the relationships of the new taxon, as well as reanalysis of *Pachyrhachis*, shows both to be related to macrostomatans, a group that includes relatively advanced snakes such as pythons, boas, and colubroids to the exclusion of more primitive snakes such as blindsnakes and pipesnakes.

The lower to middle Cenomanian (basal Upper Cretaceous) carbonates of 'Ein Yabrud near Jerusalem, deposited in a low-energy shallow marine platform environment (1), have yielded two species of fossil snakes, *Pachyrhachis problematicus* (2–4) and the new taxon reported here. Because of the presence of relatively well-developed hindlimbs and a supposedly primitive skull structure, a series of recent publications (5–7) have interpreted *Pachyrhachis* to be basal to all other snakes, indeed to represent “an excellent example of a transitional taxon” (8) linking snakes to an extinct group of “lizards,” the mosasauroids. On the basis of this pattern of phylogenetic relationships, it was claimed that snakes had a marine origin (8) and that the mosasauroid jaws provided the starting point for the evolution of the ophidian feeding mechanism (9). The transitional position of *Pachyrhachis* influenced a scenario explaining the origin and

evolution of limblessness in snakes, based on the analysis of underlying developmental mechanisms as revealed by patterns of *Hox* gene expression in *Python* (10). The basal position of *Pachyrhachis* and the putative relationships of snakes to mosasauroids were tested by a review of the character evidence and the methods of phylogenetic analysis used, and were found to be refuted by the position of *Pachyrhachis* as the sister taxon of relatively advanced (i.e., macrostomatan) snakes (11–15).

Here, we describe the second snake from 'Ein Yabrud, which is better preserved than *Pachyrhachis* in the skull and hindlimb, and which highly corroborates the macrostomatan affinities of these fossil snakes.

Haasiophis, gen. nov.

Genotypical species: *Haasiophis terrasancus*, sp. nov.

Diagnosis: A snake with a snout-vent length of 717 mm; premaxilla small and narrow, edentulous; 24 tooth positions on the maxilla, 8 on the palatine, 15 to 17 on the pterygoid, and 26 on the dentary; enamel surface of teeth distinctly striated; mandibular nerve foramen underlapped by distinct prootic process; quadrate slender and vertically oriented; coronoid process on mandible small, formed by coronoid bone only; 155 prelocaal vertebrae; at least 12 proximal caudal vertebrae with distally expanded and bifurcated lymph-

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