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Intracellular uncaging of cGMP with blue light

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Abstract

We have made a new caged cGMP that is photolyzed with blue light. Using our recently developed derivative of 7-diethylaminocoumarin (DEAC) called DEAC450, we synthesized coumarin phosphoester derivatives of cGMP with two negative charges appended to the DEAC450 moiety. DEAC450-cGMP is freely soluble in physiological buffer without the need for any organic co-solvents. With a photolysis quantum yield of 0.18 and an extinction coefficient of 43,000 M⁻¹ cm⁻¹ at 453 nm, DEAC450-cGMP is the most photosensitive caged cGMP made to date. In patch-clamped neurons in acutely isolated brain slices, blue light effectively uncaged cGMP from DEAC450 and facilitated activation of hyperpolarization and cyclic nucleotide gated cation (HCN) channels in cholinergic interneurons. Thus, DEAC450-cGMP has a unique set of optical and chemical properties that make it a useful addition to the optical arsenal available to neurobiologists.

Keywords

caged compounds; blue light; cGMP; HCN channels; coumarin; neurophysiology

Visible light penetrates the plasma membrane of living cells and so can provide non-perturbing means to study the dynamics of intracellular processes¹. Technology development has played a fundamental role in the knowledge garnered from such optical experiments². Phase contrast imaging³, along with its video rate version^{4, 5}, is recognized as a key event for live cell biology, as it allowed us to observe and record rapid changes longitudinally inside cells for the first time⁶. In order to use light to control a defined intracellular process some form of inbuilt latency needed to be introduced inside cells⁷. Photochemical protecting groups, developed by organic chemists in the 1960s⁸, were exploited by physiologists for this purpose for the first time in 1978⁹. Thus, an *ortho*-

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Author contributions. HKA synthesized DEAC450-cGMP. GCRE-D characterized the photochemical properties of DEAC450-cGMP. SZ and DJS designed and performed the electrophysiological experiments. SZ analyzed the physiology data. All authors commented upon the MS and approved the final submission.

Supporting Information: LC-MS of photochemical reaction and NMR and accurate mass spectra of newly synthesized compounds.

nitrobenzyl (NB) derivative of ATP, biologically inactive until it was irradiated by light, was introduced into red blood cells and used to initiate active transport of sodium ions in a time resolved way. This optical probe was dubbed “caged ATP” by the biologists who made it, probably unaware that “caged” was used by chemists to refer to box-like structures such as cubane¹⁰. Having demonstrated the extraordinary potential of light to control intracellular signaling^{9, 11}, every other important second messenger was caged for biology using the same *ortho*-nitrobenzyl photochemical protecting group^{7, 12}. And its continued use is testament to its abidingly useful set of properties for many types of experiments¹². But the *ortho*-nitrobenzyl chromophore, and its analogous 4,5-dimethoxy derivative (DMNB)¹³, are best photolyzed with wavelengths of light in the 350 nm range. Whilst many laboratories are equipped with such light sources, the recent wide availability of blue lasers (or LEDs) for in vivo experiments¹⁴ suggests that caged compounds that are highly active to such light would be advantageous to many biologically focused laboratories. Thus, we have recently developed a new caging chromophore that is easily photolyzed by blue light^{15–19} (430–480 nm). Called “DEAC450”, this chromophore offers excellent photochemical efficiency to excitation below 500 nm. Like cAMP, cGMP has many functions in the brain, such as mediating sensory transduction and regulating synaptic plasticity and behavior^{20, 21}. In this study we describe the synthesis and application of DEAC450-caged cGMP.

Results and discussion

The synthesis of DEAC450-cGMP (**1**) started with our previously made¹⁶ mesylate **2**, which was used to alkylate cGMP in DMF (Scheme 1). To enable this reaction to proceed in an organic solvent tri-*n*-octylamine was mixed with the free acid of cGMP and heated in DMF for 1 h. It is important to note that the conditions we used previously for the synthesis of DEAC450-cAMP (*n*-butylamine in methanol) were completely unsuccessful for cGMP, probably because the base and solvent did not enable the formation of a solubilized form of cGMP. Once we had a DMF solution of cGMP octylamine salt, we heated this at 65°C with **2** for 18 h. The pure equatorial and axial isomers could be isolated by reverse-phase HPLC in a combined yield of 14%. For the deprotection step, we treated the isomeric mixture in a dichloromethane solution with TFA for 3 h at RT to give the target caged compound **1**. Pure equatorial (**1a**) and axial (**1b**) isomers were isolated by HPLC in combined yield of 55%. The purified products were found to be soluble in aqueous buffer at pH 7.2, with solutions of at least 1 mM easily made without the use of any organic co-solvent. Consistent with reports of other coumarin-caged phosphates^{22–24}, such solutions were found to be stable frozen for periods of at least two months.

We photolyzed a solution of **1** in HEPES (40 mM, pH 7.2, with 100 mM KCl) with 473nm light and found that two major photoproducts could be seen in the HPLC chromatogram (Figure 1). The retention times and UV-visible absorption spectra from the diode array detector corresponded to the expected cGMP (**4**) and DEAC450-OH (**5**). The slight hypsochromic shift in the coumarin absorption maximum upon photolysis is similar to that reported for other caged cyclic nucleotides. Furthermore, there is subtle but clear change in the UV region of DEAC450-cGMP with release of cGMP and guanosine absorbs strongly in this region. When this reaction mixture was analyzed by LC-MS, we found that these products had the appropriate high-resolution masses (Supplemental Figure 1). These data

together show conclusively that cGMP is a major photoproduct from irradiation of DEAC450-caged cGMP.

Next, we determined the quantum yield of photolysis. We measured the photon flux with a calibrated power meter and used HPLC to monitor the extent of photolysis of the caged cGMP²⁵. A vigorously stirred solution, with an initial concentration of **1a** of 0.00465 mM, absorbed 0.5 mW of 473 nm laser light. The solution had an optical density of 0.2, which in a volume of 2 mL, corresponds to 5.6×10^{15} molecules. Illumination for 5 s (or 5.94×10^{15} photons) produced 19% photolysis, corresponding to a quantum yield of photolysis of **1a** of 0.18. Note both isomers are equally photoactive. Our photochemical data indicate that DEAC450-cGMP is the most optically efficient caged version of cGMP made so far. Table 1 summarises the properties of the published probes for comparison with DEAC450-cGMP. Importantly, our new optical probe is, uniquely, highly active in the blue region of the electromagnetic spectrum, making it chromatically complementary to many other caged compounds (e.g. MNI-Glu, DM-nitrophen, CDNI-GABA, etc.) used by neurobiologists today.

Several reports of new caging chromophores that absorb blue or green light have appeared. Organic-inorganic hybrid systems based on ruthenium-bipyridial (or “RuBi”) complexes offer the ability to cage amine bonds effectively, with a quantum yield of photolysis of 0.14 reported²⁶. RuBi does not work for acidic bonds and so many groups have explored different coumarin chromophores as visible-light absorbing photochemical protecting groups. However, even though these chromophores absorb light much more efficiently than RuBi, they seem to have much lower quantum yields, ranging from 0.005²⁷ – 0.003²⁸ to as low as 0.000001²⁹. Similar trends are seen when using the BODIPY fluorophore as a photochemical protecting group, with a caged GABA photolysis quantum yield of 0.0003³⁰. In contrast, DEAC450 acid photorelease is much more efficient, potentially making physiological experiments more practical.

We decided to test the utility of our new optical probe with a neuronal assay. We chose the well-characterized hyperpolarization activated cyclic nucleotide gated cation channel (HCN) in cholinergic interneurons in the dorsal striatum as the target for DEAC450-cGMP uncaging. The advantage of using this approach is that cyclic nucleotides gate HCN channels directly³¹. In other words, protein phosphorylation is not the primary mechanism of channel activation, thus any effects from uncaging are immediate. Furthermore, since the HCN current is inherently distinctive, as large currents are seen at very negative potentials, it could allow us to make a clear-cut measurement of rapid cGMP release. Thus, we patch-clamped cholinergic interneurons identified by their large somata and aspiny dendrites (Figure 2a) and the presence of spontaneous spikes, with an internal solution that contained 75 μ M DEAC450-cGMP. We found that irradiation with blue light (470nm LED) for 200 ms immediately before hyperpolarizing steps (–60 mV to –90 or –110 mV) caused the expected enhancement in the HCN current (Figure 2b). The HCN tail current was also enhanced. As expected³², the activation voltage-dependence of HCN channels, measured as the half-maximal activation voltage of the tail currents, was also shifted in the depolarized direction by cGMP uncaging. These effects were seen in all cells tested and were statistically significant ($n = 4$, $P < 0.05$, student’s t-test, Figure 2c–e).

cGMP regulates many intracellular signaling cascades, including cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels that are involved in many physiological processes, such as smooth muscle motility, intestinal fluid and electrolyte homeostasis, and visual phototransduction²⁰. DMNB-cGMP³³ was one of the first caged compounds used for rapid photorelease and its use showed definitively that fast changes in [cGMP] could gate visual transduction³⁴. However, it was reported³³ that this probe was somewhat hydrolytically sensitive at physiological pH. Thus, Wootton and coworkers developed³⁵ a very stable, highly soluble dicarboxy(dc)NB-cGMP caged compound with approximately the same photochemical sensitivity to the DMNB probe in the UV. Subsequently Hagen and co-workers made²² DEAC derivatives with absorption maxima that are bathochromic to the NB and DMNB chromophores (Figure 3). However, none of these optical probes are sensitive to blue light of >450nm (Table 1). Since genetically encoded actuators typically absorb in the blue³⁶ and these are widely used by neurobiologists³⁷, the development of other optical probes that are highly sensitive to light in this region could be advantageous for many laboratories. DEAC450-cGMP is an optical probe that fits this remit being the only caged cGMP that effectively absorbs blue light (Table 1). In fact, the DEAC450 chromophore actually absorbs *green* light more efficiently than the standard nitroaromatic chromophores absorb near-UV light (Figure 3). Furthermore, DEAC450 absorption *minimum* suggests this caged compound could also be a useful optically complementary probe for 2-color uncaging experiments with well-established short wavelength caged compounds^{18, 38}.

Summary

We have synthesized the first caged cGMP that is highly photosensitive to blue light. We established the efficacy of this new optical probe by uncaging in neurons in brain slices to enhance rapidly a HCN channel current in cholinergic interneurons. This new caged compound could be easily used by neurobiologists because of the availability of such blue light sources in many laboratories.

Methods

Synthesis and photochemical methods

Di-tert-butyl ((E)-3-(4-((((4aR,6R,7R,7aS)-6-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)-7-hydroxy-2-oxido-2,3,4,5-tetrahydro-4H-furo[3,2-d][1,3,2]dioxaphosphinin-2-yl)oxy)methyl)-7-(diethylamino)-2-oxo-2H-chromen-3-yl)acryloyl)-L-aspartate (3)—To a suspension of cGMP (25 mg, 72.4 μmol) in DMF (25 mL) was added tri-n-octylamine (63 μL, 145 μmol). The reaction was heated at 65 °C for one hour, at which time a clear solution was obtained. Compound **2** (45 mg, 72.4 μmol) was added to the reaction mixture, which was then heated at 65 °C for 18 h. The reaction mixture was purified by reverse phase HPLC (55% MeCN in water, 0.1 % TFA). Solvents were removed under reduced pressure to give 9 mg of **3** (14%) as a 1:1 mixture of equatorial (**3a**) and axial (**3b**) isomers as amorphous yellow solids. (**3a**): ¹H NMR (300 MHz, CD₃OD) δ 8.32 (bs, 1H), 7.83 (d, 1H, J = 15.2 Hz), 7.76 (d, 1H, J = 9.3 Hz), 7.34 (d, 1H, J = 15.2 Hz), 6.76 (dd, 1H, J = 2.4 and 9.3 Hz), 6.52 (d, 1H, J = 2.4 Hz), 6.00 (s, 1H), 5.56 (d, 2H, J = 7.8

Hz), 4.57–4.76 (m, 4H), 4.40 (dd, 1H, 9.7 and 15.6 Hz), 3.57–3.42 (m, 5H), 2.85–2.70 (m, 2H), 1.46 (s, 9H), 1.45 (s, 9H), 1.19 (t, 6H, $J = 7.0$ Hz); LCMS (ESI) m/z calc'd for $C_{39}H_{51}N_7O_{14}P$ $[M-H]^+$ 872.3232, found 872.3243. (**3b**): 1H NMR (300 MHz, CD_3OD) δ 8.30 (bs, 1H), 7.84 (d, 1H, $J = 15.2$ Hz), 7.76 (d, 1H, $J = 9.3$ Hz), 7.35 (d, 1H, $J = 15.2$ Hz), 6.83 (dd, 1H, $J = 2.4$ and 9.3 Hz), 6.56 (d, 1H, $J = 2.4$ Hz), 5.99 (s, 1H), 5.40–5.65 (m, 3H), 4.72–4.82 (m, 4H), 4.50–4.58 (m, 1H), 3.52 (q, 4H, $J = 7$ Hz), 2.75 (ddd, 2H, 6.2 Hz, 16.2 Hz and 35.1 Hz), 1.45 (s, 9H), 1.44 (s, 9H), 1.23 (t, 6H, $J = 7.0$ Hz); LCMS (ESI) m/z calc'd for $C_{39}H_{51}N_7O_{14}P$ $[M-H]^+$ 872.3232, found 872.3208. (**3a + 3b**): ^{13}C NMR (150 MHz, CD_3OD) δ 170.18, 167.68, 167.49, 160.77, 156.02, 154.56, 151.97, 146.51, 146.29, 132.23, 132.03, 127.13, 126.93, 124.68, 124.56, 114.00, 110.07, 109.88, 107.55, 96.69, 93.24, 93.02, 82.03, 81.99, 81.26, 79.13, 78.67, 72.46, 72.42, 71.83, 70.85, 70.66, 70.53, 61.89, 60.36, 50.33, 50.14, 46.67, 44.62, 37.26, 37.13, 29.55, 28.95, 27.11, 26.98, 11.57;

((E)-3-(4-(((4aR,6R,7R,7aS)-6-(2-Amino-6-oxo-1,6-dihydro-9H-purin-9-yl)-7-hydroxy-2-oxidotetrahydro-4H-furo[3,2-d][1,3,2]dioxaphosphinin-2-yl)oxy)methyl)-7-(diethylamino)-2-oxo-2H-chromen-3-yl)acryloyl)-L-aspartic acid (1)—To a solution of **3** (8.8 mg, 10.1 μ mol) in dichloromethane (5 mL) was added TFA (5 mL) and the reaction mixture was stirred at room temperature for 3 h. The solvents were removed to give 1:1 mixture of equatorial and axial isomers of **1** which were purified by HPLC (35% MeCN in water, 0.1% TFA) to give 3.9 mg of **1a** (equatorial) and **1b** (axial) in combined yield of 51 % as amorphous yellow solids. (**1a**): 1H NMR (300 MHz, CD_3OD) δ 7.67–7.86 (m, 2H), 7.28 (d, 1H, $J = 15.2$ Hz), 6.73 (d, 1H, $J = 8.3$ Hz), 6.51 (s, 1H), 5.96 (s, 1H), 5.47–5.65 (m, 2H), 4.89–5.00 (m, 1H), 4.29–4.65 (m, 3H), 3.36–3.52 (m, 4H), 2.93 (br s, 2H), 1.15 (t, 6H, $J = 6.6$ Hz). ^{13}C NMR (150 MHz, CD_3OD) δ 172.87, 167.66, 160.73, 156.05, 151.92, 146.53, 132.03, 126.95, 124.61, 114.02, 109.91, 107.48, 96.71, 79.15, 72.46, 70.90, 70.73, 60.37, 44.63, 11.59. ^{31}P NMR (243 MHz, CD_3OD) δ –5.43. LCMS (ESI) m/z calc'd for $C_{31}H_{35}N_7O_{14}P$ $[M-H]^+$ 760.1980, found 760.1965. (**1b**): 1H NMR (300 MHz, CD_3OD) δ 8.03 (br s, 1H), 7.79 (d, 1H, $J = 15.2$ Hz), 7.76 (d, 1H, $J = 9.3$ Hz), 7.28 (d, 1H, $J = 15.2$ Hz), 6.85 (dd, 1H, $J = 2.4$ and 9.3 Hz), 6.58 (d, 1H, $J = 2.4$ Hz), 5.96 (s, 1H), 5.40–5.64 (m, 3H), 4.42–4.61 (m, 2H), 3.52 (q, 4H, $J = 7.0$ Hz), 2.84–3.02 (m, 1H), 1.22 (t, 6H, $J = 7.0$ Hz). ^{13}C NMR (150 MHz, CD_3OD) δ 173.16, 167.89, 161.36, 155.94, 152.05, 146.57, 132.40, 127.25, 124.66, 113.48, 110.42, 107.53, 96.79, 93.78, 78.57, 71.77, 70.60, 62.09, 52.88, 44.81, 35.88, 25.66, 19.70, 11.67. ^{31}P NMR (243 MHz, CD_3OD) δ –3.63. LCMS (ESI) m/z calc'd for $C_{31}H_{35}N_7O_{14}P$ $[M-H]^+$ 760.1980, found 760.1959.

The quantum yield of uncaging was determined using the definition $QY = (\text{no. of molecules photolyzed})/(\text{no. of photons absorbed})$. We used a calibrated power meter (Thorlabs, catalog no. S170C) to quantify the power absorbed by a solution of DEAC450-cGMP. We irradiated solutions for different periods (5–10 s), and the extent of photolysis was analyzed in triplicate by HPLC. Concentrations were set to give an OD = 0.2 at 473 nm in a 1 cm cuvette. A 473 nm laser was used for irradiation.

Animals and brain slice preparation

Animal use procedures were approved by the Northwestern University Institutional Animal Care and Use Committee. Parasagittal slices (280 μm) were prepared from male D1-tDTomato BAC transgenic mice (P60–P90). The mice were anesthetized with a mixture of ketamine (100 mg kg^{-1}) and xylazine (7 mg kg^{-1}) and perfused transcardially with ice-cold sucrose-based cutting solution containing (in mM): 181 sucrose, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 2.5 KCl, 0.5 CaCl_2 , 7 MgCl_2 , 11.6 sodium ascorbate, 3.1 sodium pyruvate and 5 glucose (305 mOsm L^{-1}). After sectioning, slices were incubated for 40 min at 34 °C in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1 NaH_2PO_4 , 2.0 CaCl_2 , 1.0 MgCl_2 , 26 NaHCO_3 and 13.89 glucose, after which they were stored at room temperature until recording. External solutions were oxygenated with carbogen (95% CO_2 /5% O_2) at all times.

Electrophysiology and two-photon laser scanning microscopy

Individual slices were transferred to a recording chamber and continuously superfused with ACSF (2–3 ml/min , 32°C). Whole-cell voltage clamp recordings were obtained from dorsolateral striatum. Patch pipettes (3–4 $\text{M}\Omega$ resistance) were loaded with internal solution containing (mM): 115 K-gluconate, 20 KCl, 1.5 MgCl_2 , 5 HEPES, 5 EGTA, 2 Mg-ATP , 0.5 Na-GTP, 10 Na-phosphocreatine, 0.05 Alexa 568 hydrazide, 0.075 DEAC-cGMP (pH 7.25, osmolality 280–290 mOsm L^{-1}). To avoid unintended photolysis of DEAC450-cGMP, pipette solution preparation and patch clamp were carried out under red-light (630nm) or orange-light (550nm) illumination in a dark room. After patch rupture, the pipette solution was allowed to equilibrate for 8–12 minutes for filling the cell with DEAC450-cGMP. All the recordings were made using a MultiClamp 700B amplifier (Axon Instrument, USA), and signals were filtered at 2 kHz and digitized at 10 kHz.

I_h was activated by changing the membrane potential from a holding potential of -60 mV to a series of test potentials (from -70 mV to -130 mV). Each test potential was maintained for 1s. The I_h current was measured as the steady-state current at the end of the test potential minus the instantaneous current following the capacitive transient at the start of the test potential. The tail current (I_{tail}) was measured as the peak amplitude of the residual inward current evoked by returning the holding potential to -60 mV. The voltage that half-maximally activated I_h ($V_{1/2}$) was determined by fitting the tail currents to a Boltzmann sigmoidal function. Intracellular uncaging of DEAC450-cGMP was achieved by a 200ms pulse of 470nm light (pE-100, CoolLED) at a light intensity of 11.3 mW/mm^2 at the objective lens. The pre-uncaging and post-uncaging measurements were taken from a time window of 20s immediately before and after the uncaging light pulse, respectively. Off-line analysis was performed using Python and Origin 8 (OriginLab, USA).

At the end of each experiment, the identity of the recorded neuron was confirmed by visualizing Alexa568-filled cell under a two-photon Ultima laser scanning microscope (Prairie Technologies (now Bruker), Middleton, WI, USA) and 810 nm excitation (Verdi/Mira laser: Coherent). Maximum projection image of the cholinergic interneuron was acquired with $0.389 \mu\text{m} \times 0.389 \mu\text{m}$ pixels, 1 μm z-steps, and 4 μs pixel dwell time. Voltage protocols, data acquisition and imaging were performed using PrairieView 5.3 (Bruker).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Photolysis of DEAC450-cGMP with blue light.

The equatorial and axial isomers of DEAC450-cGMP were irradiated together at physiological pH. Two major products were detected by HPLC corresponding to the desired cGMP (**4**) and the “spent cage”, alcohol **5**. UV-visible absorption spectra from the HPLC diode array detector are inset. Note the slight hypsochromic shift in the coumarin absorption maximum upon photolysis and the change in the UV region of DEAC450-cGMP with release of cGMP.

Fig 2.

Intracellular uncaging of DEAC450-cGMP in striatal cholinergic interneurons enhances hyperpolarization-activated current (I_h). (a) Morphological identification of an Alexa568-filled striatal cholinergic interneuron (ChI) in a D1-Tdtomato brain slice. Note that a cholinergic interneuron is readily identifiable because it is much larger in size compared to nearby direct-pathway spiny projection neurons (dSPNs) and devoid of dendritic spines. (b) Upper, an example of voltage step protocol used to evoke I_h . Lower, sample traces showing that cGMP uncaging caused an enhancement of I_h current and tail current (I_{tail}). (c) Effect of DEAC450-cGMP photolysis on I_h current (normalized by cell capacitance). (d) Effect of DEAC450-cGMP photolysis on I_{tail} (normalized by cell capacitance). (e) DEAC450-cGMP photolysis shifted the half-maximal activation potential ($V_{1/2}$) to a more depolarized potential. Grey filled circles, individual cells; black filled circles, averaged data. All summary data are presented as mean $\pm$ SEM. Asterisks denote statistical significance ($P < 0.05$) according to paired t-test.

Fig 3.

Absorption spectra of NB, DMNB, DCAC and DEAC450 chromophores. The UV-visible absorption spectra of the four caging chromophores are displayed as their absolute molar extinction coefficient values. Green, *ortho*-nitrobenzyl; orange, 4,5-dimethoxy-2-nitrobenzyl; violet, *N,N*-dicarboxymethyl-7-aminocoumarin (DCAC); blue, DEAC450-cGMP.

Scheme 1.

Synthesis and photolysis of DEAC450-cGMP.

Reagents and conditions: (a) cGMP, tri-*n*-octylamine, DMF, 65°C, 18 h. (b) TFA, DCM, RT, 3 h. The photoproducts (**4** and **5**) were confirmed by HR-MS (Supplementary Figure 1), and their HPLC retention times.

Table

Summary of the photochemical properties of caged cGMP probes. ϵ , extinction coefficient ($M^{-1} \text{ cm}^{-1}$); ϕ , quantum yield; Bhc, bromohydroxycoumarin.

Probe	ϵ (λ_{max})	ϕ	$\epsilon \cdot \phi$	ϵ_{473}
dcNB ³⁵	500 (350)	0.24	120	0
DMNB ³³	5,000 (350)	0.05	250	0
DEAC ²²	19,100 (396)	0.26	4,966	0
DCAC ²³	18,700 (383)	0.30	5,610	0
Bhc ³⁹	15,400 (372)	0.12	1,850	0
DEAC450	43,000 (453)	0.18	7,740	40,700